NATURAL SUBSTRATES AND INHIBITORS OF CYP1 FAMILY ENZYMES

PHD THESIS SUBMITTED BY

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"It was always my established belief that most intrigues occur in politics. However I had to reconsider after being familiar with universities."

Harold Wilson, Prime minister of Great Britain 1964-70, 1974-76.

The enzymes CYP1A1 and CYP1B1 have been shown to be overexpressed in a wide variety of tumors. The enzyme CYP1B1 has been shown to convert a cancer preventative agent (resveratrol) to a compound with antileukemic activity (piceatannol). Based on these findings interactions of CYP1 family enzymes with cancer preventative flavonoids, that have structure similar to resveratrol, were investigated. The flavonols quercetin, myricetin and kaempferol and the flavones chrysin, eupatorin and diosmetin inhibit the dealkylation of 7-ethoxyresorufin to resorufin by CYP1B1, and to a lesser extent by CYP1A1 (with the exception of chrysin) and CYP1A2. The flavones diosmetin, eupatorin, sinensetin, genkwanin, scutellarein and chrysin are substrates for CYP1 family enzymes.

Furthermore the metabolism of natural flavones and their cytotoxic action in MDA-MB 468, MCF7 human breast adenocarcinoma and MCF10A human breast cells, as well as the ability to inhibit the progression of these cells through the phases of the cell cycle was evaluated. The flavones diosmetin, genkwanin and eupatorin were shown to be metabolised in MDA-MB 468 cells and MCF7 cells pretreated with 10nM TCDD for 24 hrs but not in MCF10A cells or MCF7 cells with no TCDD treatment. The latter compounds along with other structurally related flavones, were shown to inhibit the growth of MDA-MB 468 and MCF7 cells, while having minor effects on the growth of MCF10A cells (with the exception of baicalein). The flavones eupatorin and cirsiliol caused G_2/M arrests in MDA-MB 468 cells at 48 hr and diosmetin and sinensetin caused G_1 arrests in the same time period.

Finally the ability of natural flavones to induce the expression of CYP1 family enzymes in MCF7 cells was investigated. The flavones diosmetin, genkwanin, eupatorin,

eupatorin-5-methyl ether and cirsiliol induced CYP1 enzyme expression in MCF7 cells after 24 hr treatment, as evident by increased EROD activity. Eupatorin and cirsiliol caused an increase in *CYP1B1* and *CYP1A1* mRNA levels, as shown by RTPCR.

Overall the data confirm the hypothesis that CYP1B1 and CYP1A1 can act as tumour suppresor enzymes, via metabolic activation of naturally occuring flavonoids. Thus the results provide a novel model on the mechanism by which selected flavonoids exert their chemopreventative action.

CONTENTS

ABBREVIATIONS

AAF: 2-Acetylaminofluorene

AhR: Aryl hydrocarbon receptor

APS: Ammonium persulfate

ARNT: Aryl hydrocarbon nuclear translocator

CV: Coefficient of variation

CYPs: Cytochrome P450s

D1: 6-hydroxy luteolin

D2: Unidentified metabolite of diosmetin

DMSO: Dimethyl sulfoxide

dNTPS: deoxynucleotide triphosphates

DTT: dithiothreitol

E1: Unidentified metabolite of eupatorin

E2: Second unidentified metabolite of eupatorin

EDTA: Ethylenediamine tetraacetic acid

ER: Ethoxy resorufin

EROD: 7-ethoxy resorufin-O-deethylase

EtBr: Ethidium Bromide

FACS: Fluoresence activated cell sorting

FCS: Fetal calf serum

G1: Unidentified metabolite of genkwanin

G2: Second unidentified metabolite of genkwanin

GST: Glutathione *S* transferase

HIF1 α: Hypoxia inducible factor 1α

HIF1β: Hypoxia inducible factor 1β

HMECs: Human mammary epithelial cells

HPLC: High performance liquid chromatography

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide

NADPH: Nicotine adenine dinucleotide phosphate

PAGE: Polyacrylamide gel electrophoresis

PAHs: Polycyclic aromatic hydrocarbons

PBS: Phosphate buffered saline

PI: Propidium iodide

RTPCR: Reverse transcriptase polymerase chain reaction

SDS: Sodium dodecyl sulfate

TAE: Tris-acetate-EDTA

TBE: Tris-borate-EDTA

TCDD: 2,3,7,8-tetrachlorodibenzo-p-dioxin

TEMED: *N-N'*-tetramethylethylenediamine

Tween: Polyoxyethylenesorbitan monoeleate

1.INTRODUCTION

1.1 Cancer and cancer therapy

Cancer is a disease of abnormal gene expression, which results when the controls of normal cell growth break down. The progression of a normal cell to a cancer cell is a multistep process and is called carcinogenesis. Evidence for this finding has been provided from conditions that represent intermediate stages, where only some of the cell cycle controls have been disrupted. Carcinogenesis is caused mainly by environmental factors, such as oncogenic viruses and certain chemicals. This results to mutations in the DNA sequence of the cells.

Certain sets of genes, which are called oncogenes, have been found to be involved in carcinogenesis and their mutated versions account for corresponding oncogenic proteins, which promote the formation of cancer. Another set of genes called tumour suppressor genes protect the cell from accumulating mutations in their normal state. When inactivated, these genes lead to carcinogenesis, because their normal function is disrupted.

Cancer therapy includes radiation, surgery and chemotherapy. Initial approaches of chemotherapy have been found to be extremely cytotoxic and ineffective due to severe side effects. Cytotoxic chemotherapeutic agents include antimetabolites, which are agents that disrupt DNA synthesis, alkylating agents, which cause cross-linking between the DNA strands and natural products such as the vinca alkaloids, which cause disruption of the mitotic spindle during mitosis. Targeted therapy has emerged over the last 10 years to limit the severe side effects, caused by cytotoxic chemotherapy, with the most promising molecules being inhibitors of tyrosine kinase enzymes, which are involved in cell signalling.

1.1.1 Cancer epidemiology

In the year 2000 cancer, was diagnosed in 10 million people worldwide and caused 6.2 million deaths, an increase of about 22% since 1990 (Ferlay et al., 2001). Cancer causes 10% of all deaths worldwide and is second only to cardiovascular disease as the main cause of death in developed countries (Ferlay et al., 2001). Worldwide there are about 22 million people living with cancer at any one time (Ferlay et al., 2001). The number of cases of cancer worldwide is predicetd to increase by 5 million to 15 million new cases each year by 2020 (www.who.in/whosis). The two most common cancers worldwide are lung and breast cancer, which account for 12% and 10% of all cancers diagnosed respectively (Ferlay et al., 2001).

1.1.2 General properties

In contrast to free living cells such as bacteria, the cells of a multicellular organism are commited to collaborate with each other. Therefore, growth and development of cells and the maintenance of cell population sizes in such an organism must be subject to various types of controls. Cancer cells have lost these controls. Cancer cells are distinguished by the fact that they reproduce against the normal restraints and then invade the areas normally reserved for other cells. It is the combination of these two features that makes them particularly dangerous. A tumour or neoplasm is a relentessly growing mass of abnormal cells (Knowles and Selby, 2005). The tumour is characterised as benign if the neoplastic cells remain clustered together and as malignant if its cells can invade surrounding tissue. Metastasis is the ability of the cancer cell to spread from its site of origin to other sites of the body and form secondary tumours (Knowles and Selby, 2005).

In order for cancer cells to metastasize they must loosen their adhesion to their neighbour cells, break through the basal lamina, enter the circulation by invading the blood capillaries, travel through the bloodstream, adhere to a capillary wall and finally proliferate in the new environment. The final steps are the most difficult and only a small portion of cancer cells (less than one in a thousand) will survive to form metastatic colonies (Figure 1.1).

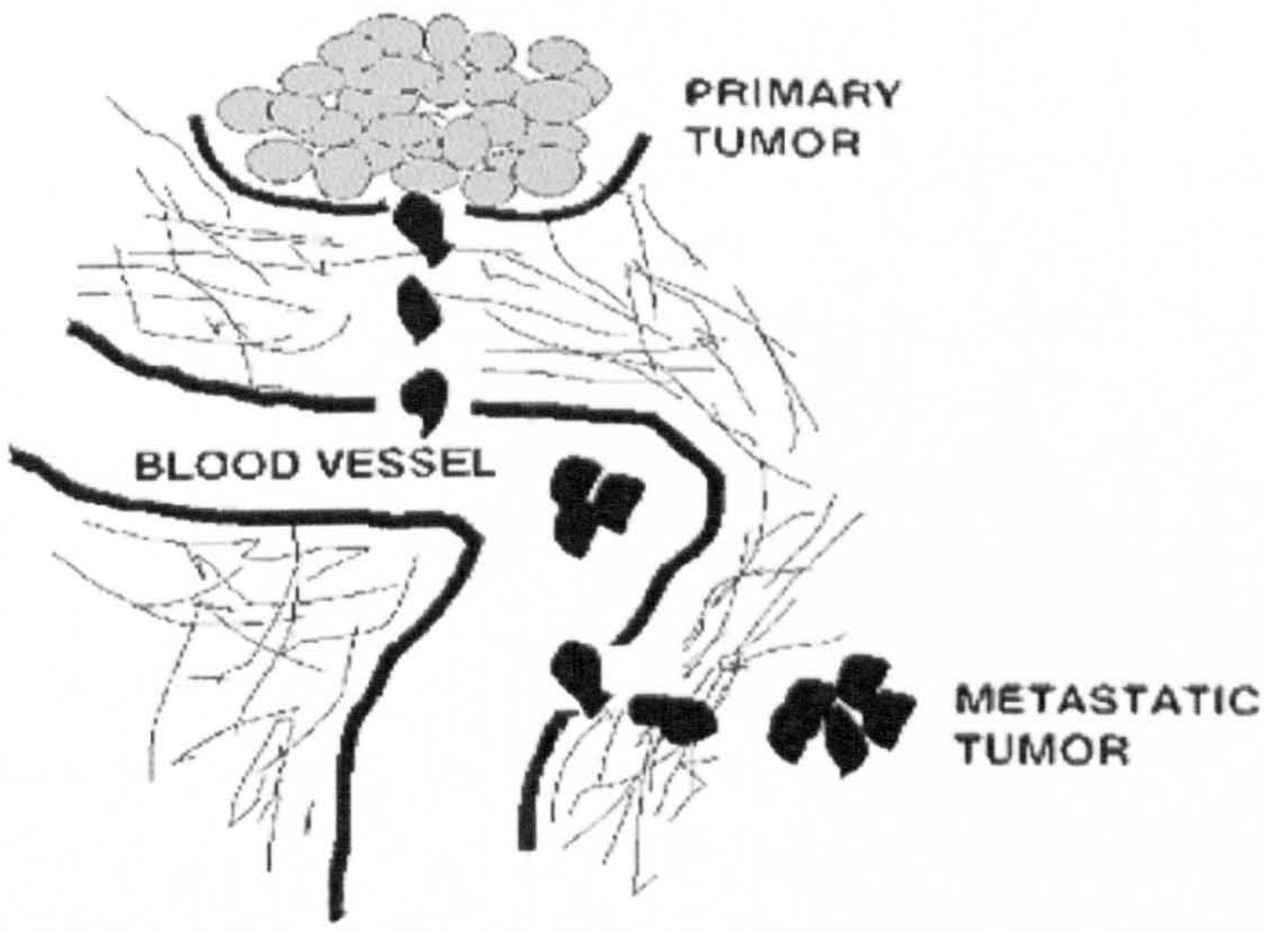


Figure 1.1 Steps in the process of metastasis, by which cells from a primary tumor travel through the bloodstream to a distant site of the body.

1.1.3 Types of cancer

Tumors are described by a generic name, which specifies the general tissue of origin (Knowles and Selby, 2005). Carcinomas, are the cancers that arise from epithelial cells,

whereas sarcomas are those which originate from muscle cells. Leukemias are derived from hemopoietic cells and there is also another broad category which involves cancers of the nervous system, such as retinoblastoma, astrocytoma etc. About 90% of human cancers are carcinomas, because of the exposure of epithelial cells to various factors that favour the development of cancer and perhaps because epithelial cells undergo most of the body's cell proliferation. Table 1.1 illustrates the major types of cancer and their incidence of death worldwide.

Table 1.1 Cancer Incidence and Mortality in the world, 2001. Adapted from Ferlay et al. ,2001

Type of cancer	New Cases per Year	Deaths per year
Colon and rectum	9%	8%
Stomach	9%	10%
Liver	5.6%	9%
Lung	12%	18%
Breast	10%	6%
Prostate gland	5.4%	3%

1.1.4 Development of cancer

Most cancers derive from a single abnormal cell, and even when a tumour has formed metastases, its origin can be traced to a single primary tumour, derived from an aberrant cell, that is a single cell that has a heritable change. A cell can become aberrant due to a

genetic or an epigenetic change. In the first case an alteration to the cells DNA sequence occurs, and in the second, a change in the pattern of gene expression occurs without requiring a change in the DNA sequence. Mutations can also occur in homeotic genes, i.e. genes that regulate concerted expression of a range of other genes. Most cancers are initiated by a genetic change and the cases which fall in the second category are rare. This genetic change is caused in most of the cases by environmental factors, since a single mutation is not enough to convert a normal cell to cancerous one. If this was the case and considering the fact that mutations occur spontaneously at a fixed rate per cell division, as a result of the accuracy of DNA replication and repair, then the chance of developing cancer in any given year would be independent of age. However epidemiological studies have shown that the incidence of cancer increases exponentially with age as shown in Figure 1.2 (Knowles and Selby, 2005). This due to the phenomenon of tumour progression, whereby an initial mild disorder of cell behaviour evolves to cancer. Hence cancer can be characterised as an evolutionary process, which involves a large element of chance, increased mutation rate and natural selection and usually takes many years to develop.

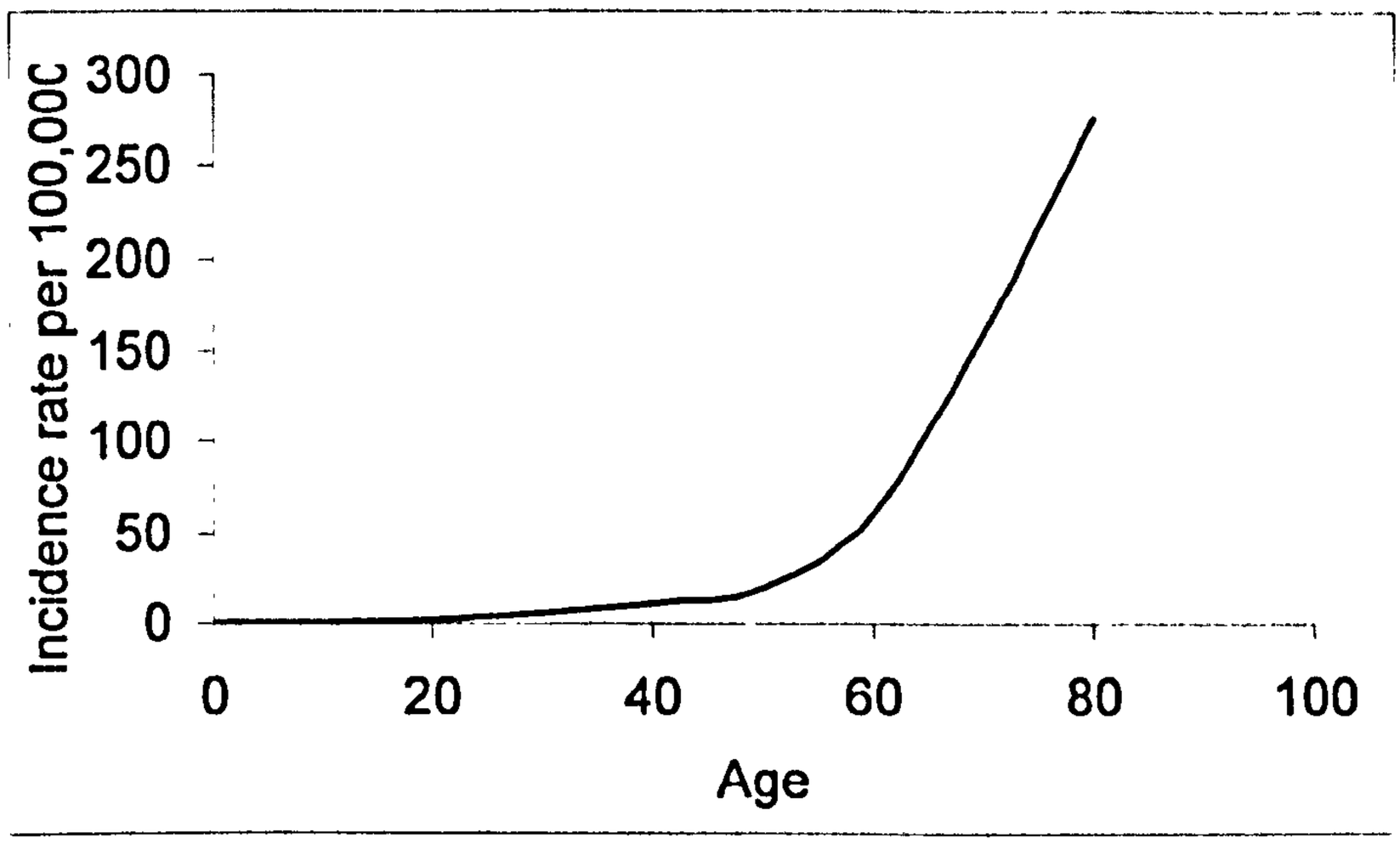


Figure 1.2 Cancer incidence as a function of age. The number of newly diagnosed cases of colon cancer in men in England in in 1999 is plotted as a function of age. Adapted from Knowles and Selby, 2005

1.1.5 Molecular genetics of cancer

1.1.5.1 General terminology

It has been shown that changes in a very small set of genes are responsible for the deranged cell behaviour observed in cancer. In recent years, major progress has been made in identifying the genes which are mutated during carcinogenesis. The genes which are responsible for causing cancer are named oncogenes. Oncogenes are mutated versions of other genes, called proto-oncogenes. This type of mutation has a dominant effect (only one copy of the gene is required to cause change in the cell behaviour) and makes the gene overactive (Chin and DePinho, 2000). A second category of genes, which cause cancer when mutated are the tumour suppressor genes. These genes act in a fundamentally different way from oncogenes. When they become inactive as the result of

mutations that eliminate their normal activity, the disappearance of their encoded proteins, causes other genes to become oncogenic (Knudson, 1996). The normal versions of these genes inhibit a normal cell from entering mitosis and cell division. Unlike oncogenes both alleles of a tumour suppressor gene need to be inactivated, hence they act in a genetically recessive fashion (Knudson, 1996).

1.1.5.2 Role of viruses in the development of cancer

About 13% of human cancers, in the whole world, are thought to arise by mechanisms that involve viruses (www.who.in/whosis). Mutations in proto-oncogenes can be triggered by viruses, which have played a remarkable part in the development of cancer and in the understanding of the key mechanisms which cause it. Retroviruses, are viruses that insert their RNA into their host (Coffin et al., 1997). The enzyme reverse transcriptase transcribes their RNA to cDNA, which then insegrates with the host's genome (Coffin et al., 1997).These viruses can act as vectors for oncogenes (Coffin et al., 1997).

Oncogenic effects are caused by retroviruses, in a process called insertional mutation, in which the altered genome is inherited by all the progeny of the original host cell (Varmus, 1988). Random insertion of the DNA copies of the viral RNA in the host's genome occurs as a part of the normal retroviral life. Another way in which retroviruses can cause mutation of a proto-oncogene is by expressing it in excess under the control of powerful promoters and enhancers in the viral genome.

The most striking example is the Rous sarcoma virus. The gene responsible for sarcomas was found in a virus called Rous sarcoma virus and was designated as v-src. By DNA-

DNA hybridisation, a sequence very similar to v-src and designated c-src was found in the vertebrate genome (Martin, 1970). This gene is the actual proto-oncogene which was picked up accidentally from the virus and had undergone mutation in the process.

However the predominant viruses, which are characterised as oncogenic are DNA viruses. Evidence for this suggestion has been given by epidemiological studies (Table 1.2 presents an overview of some of the viruses associated with human cancers). For example the incidence of liver cancer is high in areas where hepatitis-B infections are also common. The virus can be responsible for only one of the steps in the progression of cancer and other environmental factors can also be involved. Although DNA viruses incorporate their DNA into the host's genome and can possibly cause a mutation of a proto-oncogene, the resulting combined gene is a part of the viral genome and plays no role in the normal host cell (Knowles and Selby, 2005). Unlike retroviruses, DNA viruses act not by activating a proto-oncogene into an oncogene, but by interfering with the tumor suppressor genes function and thereby allowing cells with accumulated mutations to enter cell division (Knowles and Selby, 2005). Papillomaviruses contain genes which, when transcribed produce viral proteins that bind to protein products of key tumour suppressor genes of the host cell putting them out of action, and which have been implicated in malignant tumours such as uterine cervix cancers (Doorbar 2006).

Table 1.2 Viruses associated with human cancers. Adapted from Alberts et al., 1994

Virus	Associated Tumors	Areas of High Incidence
DNA viruses		
Papillomavirus (different types)	Carcinoma of uterine cervix	Worldwide
Hepatitis-B virus	Hepatocellular carcinoma	Africa
Epstein-Barr virus	Burkitt's lymphoma	Africa, New Guinea
RNA viruses		
Human T-cell leukemia virus type I (HTLV-I)	Adult T-cell leukemia/lymphoma	Japan
Human immunodeficiency Virus (HIV-1)	Kaposi's sarcoma	Africa

1.1.5.3 Tumour suppressor genes

The most important tumour suppressor genes are the Retinoblastoma (*Rb*) and the *p53* gene. The *Rb* gene is located on the long arm of chromosome 13, and it was the first tumour suppressor to be isolated (Sherr and McCornick, 2002). Originally this gene was critical for childhood tumours to develop from precursor cells in the immature retina. However *Rb* is involved in other types of tumours and although retinoblastoma is rare, loss of *Rb* has been implicated in osteosarcoma, soft tissue sarcoma, bladder carcinoma and breast carcinoma (Sherr and McCornick, 2002). In all of them, loss of heterozygosity (loss of heterozygous combination of maternal and paternal alleles, as a result of deletion or point mutation) seems to be important for the progression towards malignancy (Sherr and McCornick, 2002). The gene acts by producing Rb protein which can shuttle between an unphosphorylated and phosphorylated state. It remains unphosphorylated for cells that do not undergo cell division. In this state, Rb protein prevents DNA replication by

inhibiting transcription of proto-oncogenes. Mutation of *Rb* sets the cell free from this restraint and it has been shown that deletion of amino acids (703-737) in exon 21 of the gene produces Rb proteins which are unable to function normally (Linardopoulos et al., 1993).

However the gene that really puts tumour suppressors on the map is the one called *p53*. Deletions in this gene have been shown to be associated with more than 50% of all human cancers. The gene is located on the short arm of chromosome 17 and encodes for a 52kDa protein, which is required to bind to another regulatory gene and induce its transcription (Vousden and Lu, 2002). The product which is a 21kDa (Figure 1.3) binds with protein kinases and blocks the cell from entering the S phase of the cell cycle. *P53* seems to be expressed at high levels as a response mechanism in cells which are exposed to environmental factors that can cause cancer (Vousden and Lu, 2002). Hence loss of *p53* can aid the development of cancer first by removing the cell cycle block and allowing cells to continue proliferation and second allowing further mutations to occur when these cells divide (Vousden and Lu, 2002). Because of its importance mutations in *p53* develop at the later stages of tumour progression and only when this gene's function is disabled can cells give rise to cancer (Figure 1.4).

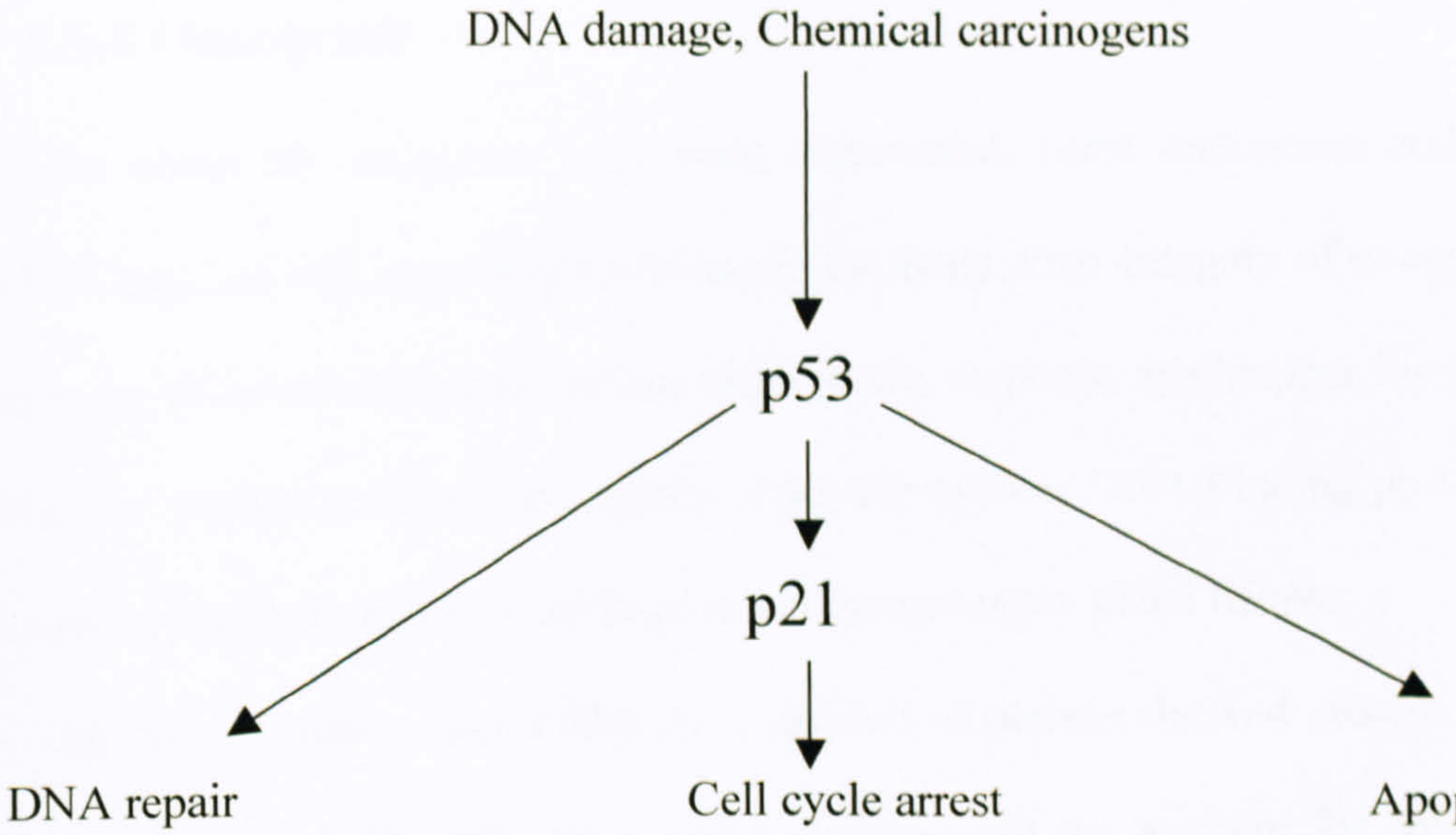


Figure 1.3 P53 maintains the genome integrity by repairing DNA inducing apoptosis or cell cycle arrest through p21.

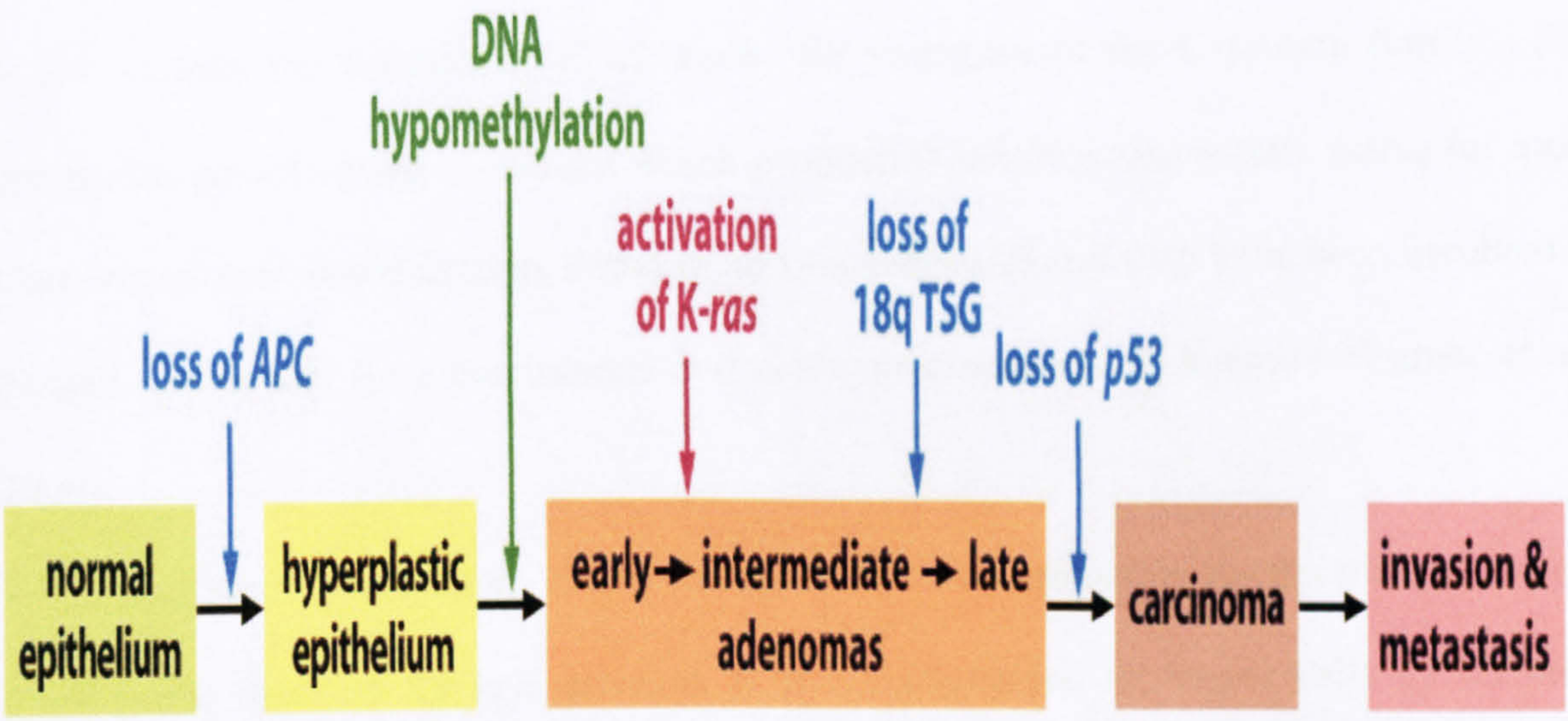


Figure 1.4 Sequence of genetic changes in the development of a colorectal carcinoma. The identity of the tumor suppressor gene 18q TSG remains unclear and so does the precise contribution of DNA hypomethylation to tumor progression. Adapted from Weinberg, 2006.

1.1.5.4 Oncogenes

So far about 60 oncogenes have been discovered. Most oncogenes code for proteins which regulate cell signalling pathways in the body. One category of oncogenes seems to code for proteins related to various steps in the response mechanism for growth factors, whereas another group of oncogenes codes for nuclear DNA binding proteins that act as transcription factors. The most important oncogenes are listed below:

– The *sis* oncogene which codes for a subunit of platelet-derived growth factor and the *fms* oncogene which codes for a mutated version of the receptor for colony-stimulating factor-1, a growth factor that stimulates bone marrow cells during blood cell formation (Turner et al., 2000).

– The various *ras* oncogenes which code for members of the G-protein family. *Ras* oncogenes possess point mutations which produce G-proteins that remain active for more than normal (discussed in more detail in section 1.1.5.6.2) and they have been involved in thyroid, colon and lung carcinomas and acute myelogenous leukaemias (Turner et al., 2000).

– The *myc* gene is located on chromosome 8 and it is responsible for the transcription of other genes required for cell division. It is overexpressed in cancer cells by different mechanisms either under the influence of a viral enhancer or by translocation of the coding sequence to another chromosome. Overexpression of *myc* oncogenes has been associated with breast and lung carcinomas and neuroblastoma (Turner et al., 2000).

– The *erbA* and *erbB* oncogenes which code for truncated versions of the epidermal growth factor receptor. Expression of *erb* oncogenes has been reported in breast and ovarian carcinomas.

1.1.5.5 Chemical carcinogenesis

The evidence that chemicals can induce cancer in humans has been accumulating for more than two centuries. The first observation was made by John Hill, who in 1761 noted that nasal cancer occurred in people who used snuff excessively (reviewed by Ruddon, 1995). Skin cancer was also observed to be occurring in people who had spent their childhood as chimney workers (1775) and in workers whose skin was in continous contact with tar and paraffin oils (1895) (reviewed by Ruddon, 1995). These observations led to attempts to induce cancer in animals with chemicals. The first succesful attempt was made in 1915, when Yamagina and Ichikawa induced skin carcinomas by the repeated application of coal tar to the ears of rabbits. This led to the search for the active carcinogen in coal tar and to the conclusion that the carcinogenic agents in tars are the polycyclic aromatic hydrocarbons. The list of known carcinogenic chemicals expanded in the 1940s, when other carcinogenic compounds were identified, such as 2-acetyl aminofluorene, halogenated hydrocarbons, urethane, beryllium salts and certain anticancer alkylating agents. Since the 1940s, various intercalating agents, asbestos, nickel, chromium compounds and nitrosamines have been added to the list.

Several studies on the reactions of carcinogens with cellular macromolecules have led to the conclusion that most of these interactions resulted from covalent bond formation between an electrophilic form of the carcinogen and nucleophilic sites in proteins (sulfur, oxygen and nitrogen atoms in cysteine, tyrosine and histidine respectively) and nucleic acids (purine or pyrimidine ring nitrogens and oxygens)(reviewed by Ruddon, 1995). The parent compound in these type of experiments, had to be frequently incubated with liver homogenates. This led to the realisation that metabolic activation of certain carcinogens

was required to produce the ultimate carcinogen that reacts with crucial molecules in the cells. Carcinogens not included in the above category are the very chemically reactive alkylating agents, which are activated in aqueous solution at physiological pH and the agents that intercalate with the DNA double helix.

1.1.5.5.1 Donor of Alkyl groups

This group contains the dialkylnitrosamines, dialkylhydrazines, aryldialkyltriazines, alkylnitrosamides and alkylnitrosimides (reviewed by Ruddon, 1995). The alkylnitrosamides can directly interact with H_2O or cellular nucleophillic groups. The alkylnitrosamines, alkylhydrazines and alkyltriazines are metabolically activated by the mixed function oxidase system (described in detail in section 1.3) in the ER of cells. The monoalkyl derivatives undergo a non-enzymatic conversion to monoalkyldiazonium ions that donate an alkyl group to cellular nucleophillic groups in DNA, RNA and protein (Figure 1.5).

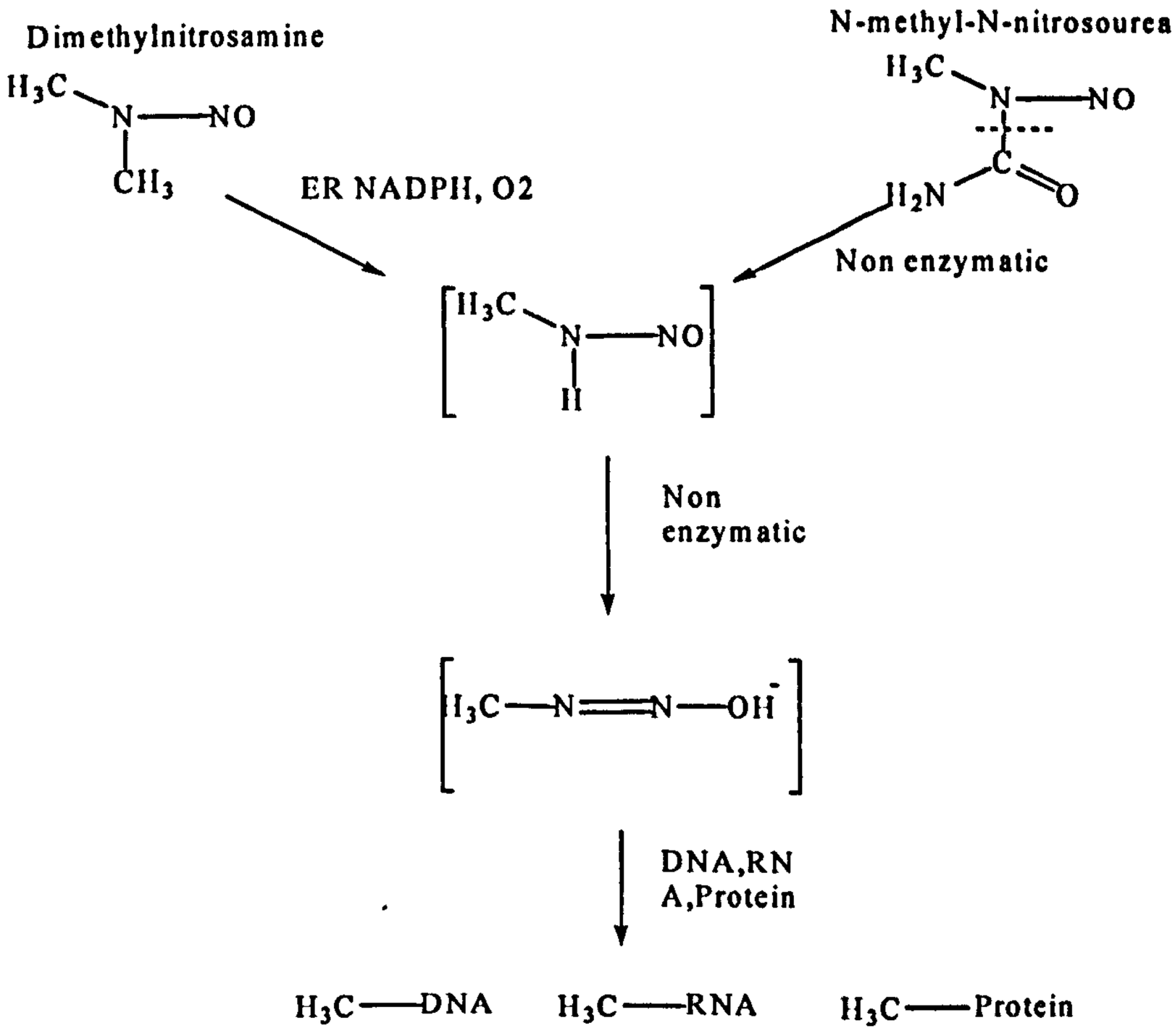


Figure 1.5. Schematic representation of nitrosamine activation adapted by Ruddon, 1995.

AAF (2-Acetylaminofluorene) in 1960 was shown to be converted to a more potent carcinogen *N*-hydroxy-AAF after the parent compound was fed to rats (reviewed by Ruddon, 1995) (Figure 1.6). Subsequent studies showed that *N*-hydroxy AAF is converted in rat liver to a sulfate *N*-sulfonoxy AAF which reacts with nucleic acids and proteins and appears to be the ultimate *in vivo* carcinogen (reviewed by Ruddon, 1995).

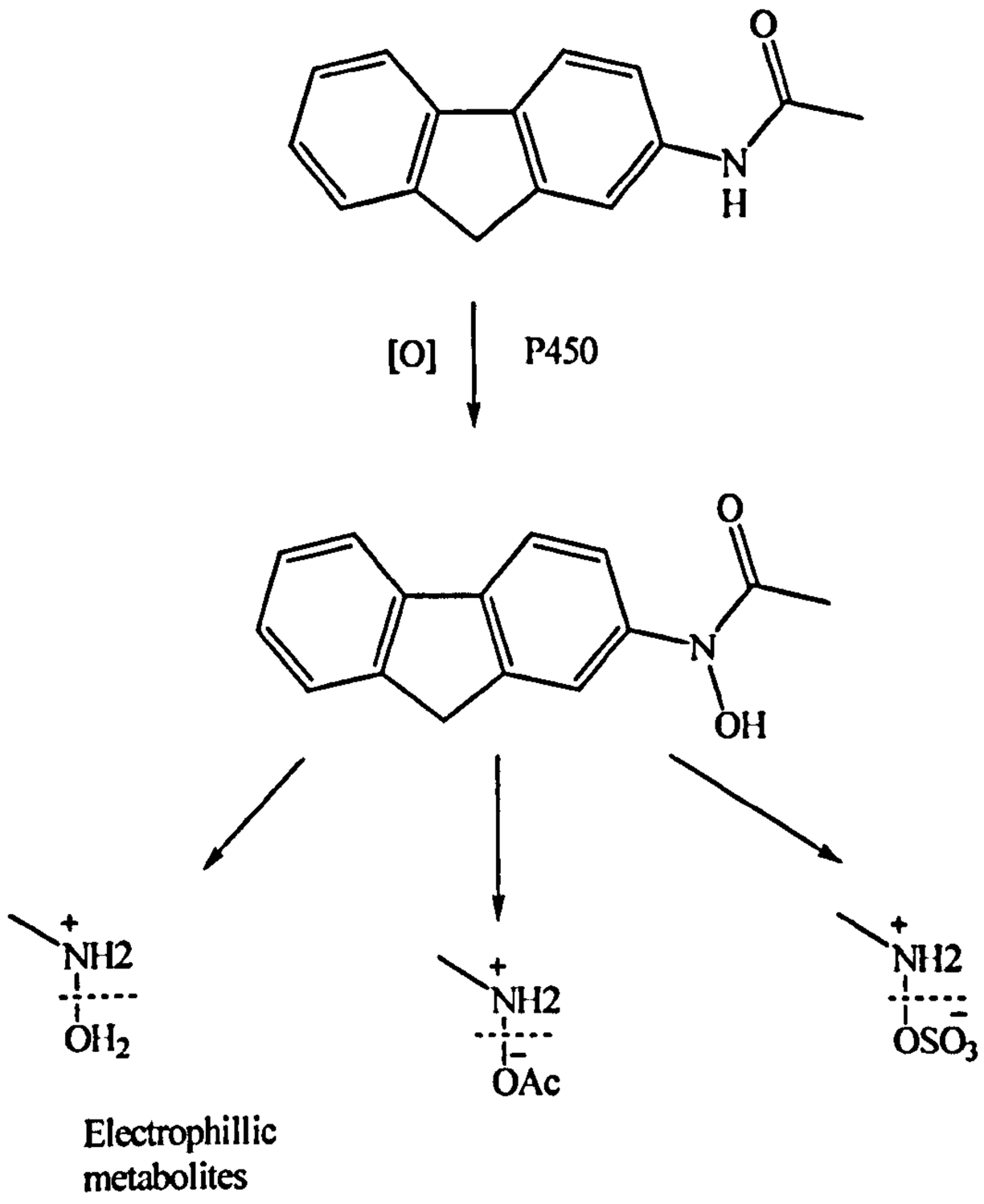


Figure 1.6. Metabolic activation of AAF adapted from Ruddon, 1995

Metabolic activation of other aromatic amines leads to formation of positively charged nitrenium ion that is formed from naphthylamine and aminobiphenyl compounds and is implicated in urinary bladder cancer in humans (reviewed by Ruddon, 1995). The mechanism of activation involves hydroxylation in the liver to a hydroxylamine derivative and subsequent glucuronidation (reviewed by Ruddon, 1995). The glucuronide conjugate is excreted in the urine, where the acidic pH converts it back to hydroxylamine, which rearranges to form the nitrenium ion, which in turn binds to macromolecules (Figure 1.7).

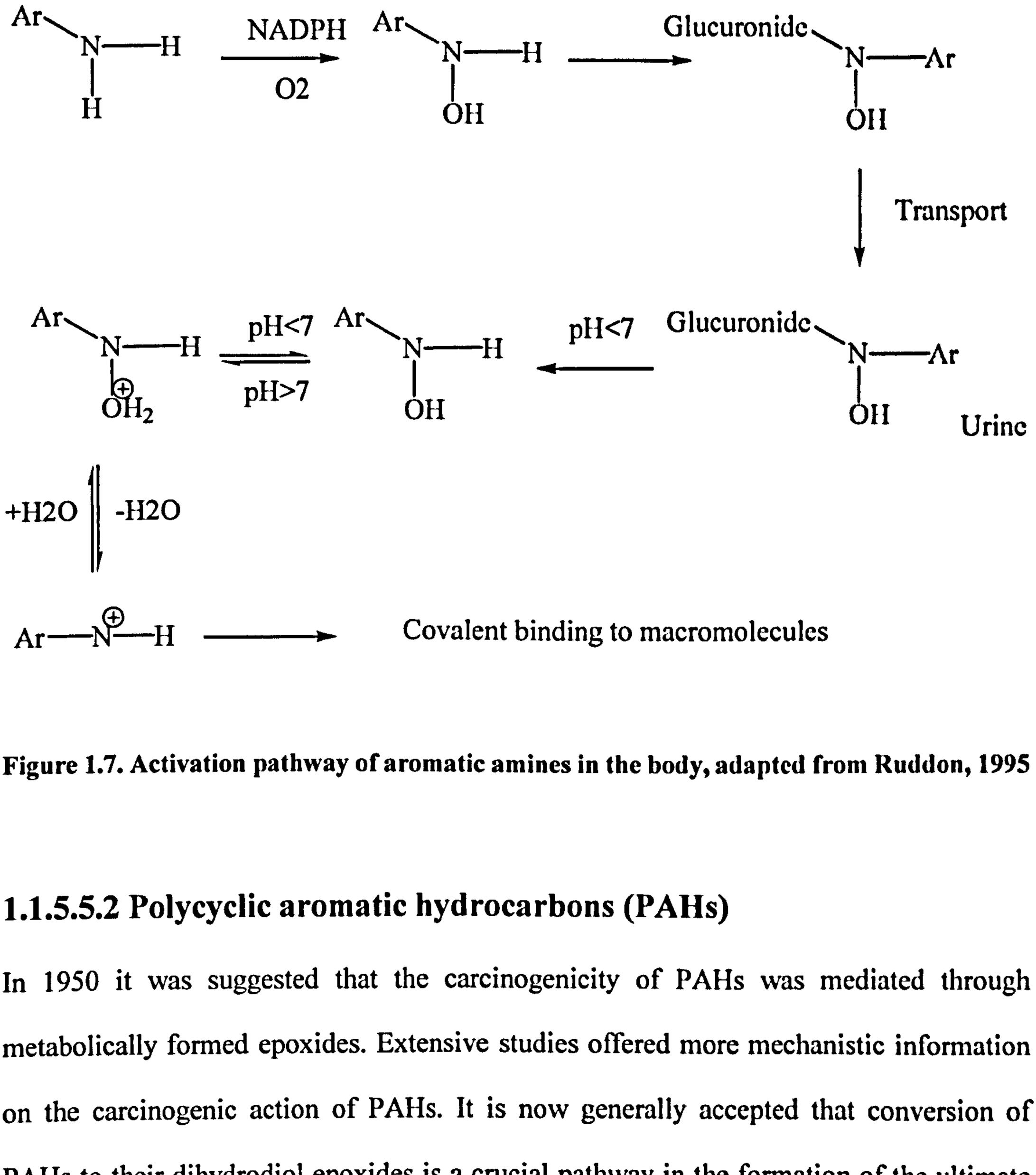
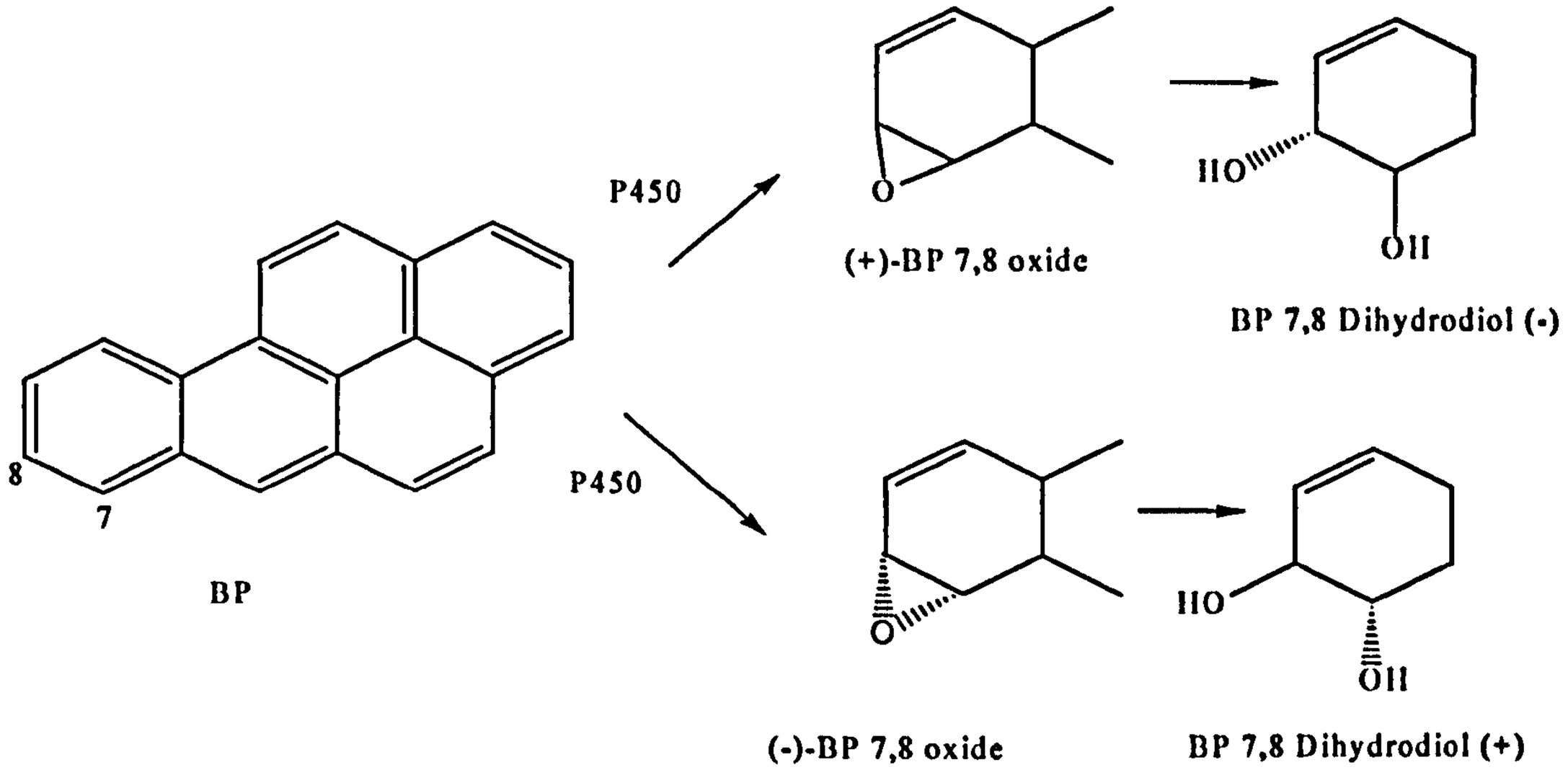


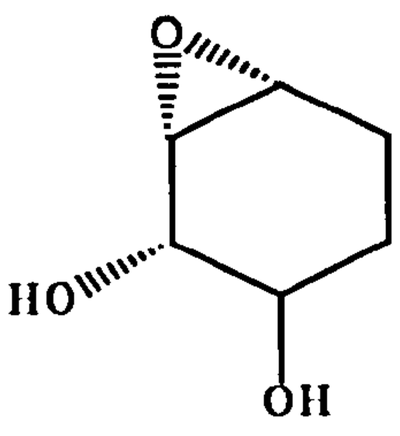
Figure 1.7. Activation pathway of aromatic amines in the body, adapted from Ruddon, 1995

1.1.5.5.2 Polycyclic aromatic hydrocarbons (PAHs)

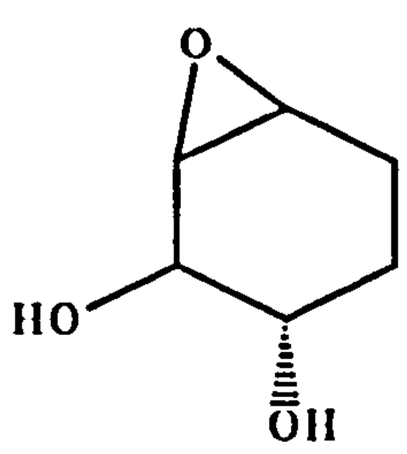
In 1950 it was suggested that the carcinogenicity of PAHs was mediated through metabolically formed epoxides. Extensive studies offered more mechanistic information on the carcinogenic action of PAHs. It is now generally accepted that conversion of PAHs to their dihydrodiol epoxides is a crucial pathway in the formation of the ultimate carcinogens. 7β,8α-dihydroxy-9α, 10α-epoxy-7,8,9,10-tetrahydroxybenzo[α]pyrene has been shown to be the ultimate mutagenic and carcinogenic metabolite of benzo[α]pyrene (reviewed by Ruddon, 1995). Further studies tested the mutagenicity of benzo[α]pyrene metabolites in mice. It was found that diol epoxides of benzo[α]pyrene (7,8 diol-9,10 epoxides) were potent mutagens as well. They could induce formation of lung adenomas-

adenocarcinomas, liver tumors and lymphomas when injected in newborn mice (reviewed by Ruddon, 1995). It was shown that benzo[α]pyrene (BP) 7,8 dihydrodiol and benzo[α]pyrene (BP) 7,8 diol-9,10 epoxide were respectively 15-fold and 40-fold more active than benzo[α]pyrene itself (reviewed by Ruddon, 1995). Diol was termed as a proximate carcinogen with the diol epoxide being the ultimate.





7,8-Diol-9,10 epoxide-2 + BP



7,8-Diol-9,10 epoxide-2 -BP

Figure 1.8. Mutagenic metabolites of benzo[α]pyrene. 7,8,9,10 epoxide-2(+) binds preferentially to DNA with 20-fold greater potency than 7,8,9,10 epoxide-2(-) adapted from Ruddon, 1995

1.1.5.5.3 DNA adduct formation

Reaction of DNA with chemical carcinogens is the most simple mechanism explaining malignant transformation, which is caused by a heritable change. Nitrosamines react with the bases of DNA and form *N-7* and *O-6* guanine derivatives. Other aromatic amines, such as *N*-methyl-4-aminobenzene produce C-8 substituted guanine residues as their primary DNA adduct (Neidle, 1979).

Various mechanisms exist by which the DNA adducts lead to cancer initiation. In some cases, such as PAHs, the flat planar ring stabilises an intercalation reaction with the stacked bases of the double helical DNA, leading to a frameshift mutation during DNA replication after the point of intercalation (Knowles and Selby, 2005). DNA adducts can create a base transition mutation, due to misplacement of their alkyl groups with wrong base during DNA replication (Knowles and Selby, 2005). Moreover many of the base adducts, which involve modifications of N-3 or N-7 positions on purines can induce an instability in the glycosidic bond between the base and the sugar, which in turn undergoes cleavage by DNA glycosylase, resulting in loss of the base and creation of an apurinic site in DNA (Knowles and Selby, 2005). This site can be filled with any base during DNA replication, resulting in a base transition or a base transversion. Further research on the interaction of carcinogens with DNA has revealed that the specificity of carcinogen binding is determined to some extent by the base sequence of DNA. For example benzo[α]pyrene diol epoxide preferentially binds in a 300-base-pair sequence 5′ to the RNA cap site of chicken β-globin DNA (reviewed by Ruddon, 1995). This demonstrates that the carcinogen binds to areas most active in gene transcription.

1.1.5.6 Epidermal growth factor receptors, MAPKs, Ras and cancer

The epidermal growth factor receptor (EGFR) family comprises four members: EGFR/ErbB1, HER2/ErbB2, HER3/ErbB3 and HER4/ErbB4. Each receptor contains an extracellular cysteine-rich domain and an intracellular region where autophosphorylation occurs upon binding with the ligand. These receptors can be coexpressed in various combinations, where they form homo- or heterodimers. The ligands include EGF, TGF-α, heparin binding EGF like growth factor (HB-EGF), betacellulin and others (Guillemard and Saragovi, 2004). The above ligands can bind to EGFR, HER3 and HER4 but to date no cognate natural ligand has been identified for HER2. HER2/neu was first identified as the transforming gene in a rat neuroblastoma cell line (Guillemard and Saragovi, 2004). Overexpression of HER2 has been shown in a panel of human cancers clinical data showed a correlation between HER2 overexpression and reduced survival of patients due to increased metastatic potential (Guillemard and Saragovi, 2004). EGFR is also frequently overexpressed in a number of human cancers and its hyperstimulation enhances cellular proliferation, decreases apoptosis and promotes tumour cell motility and invasion. As in the case of HER2 high levels of EGFR expression correlate with high metastatic rate. Moreover increased rate of tumour formation in non small cell lung, bladder, cervical, kidney and ovarian cancer has been noticed along with EGFR overexpression in those tissues (Guillemard and Saragovi, 2004).

1.1.5.6.1 MAPK pathways

Various extracellular signals are converted into intracellular responses through serial phosphorylation cascades, catalysed by a group of serine/threonine kinases, called the

MAPKs (mitogen activated protein kinases). Three distinct but parallel MAP kinase cascades (ERK, JNK and p38) have been identified in mammalian cells and each one consists of a module of three kinases: a MAPK kinase kinase (MAPKKK), which phosporylates a MAPK kinase (MAPKK), which in turn phosphorylates a MAPK. The ERK (extracellular signal-regulated kinase) is activated by mitogenic stimuli, such as growth factors (e.g. EGF) which bind to a growth factor receptor (e.g. EGFR), which is a tyrosine kinase enzyme. The JNK (JUN-N terminal kinase) and p38 MAPK are activated by environmental stress stimuli, such as UV and ionising radiation. Once activated these three MAPKs (ERK, JNK and p38) can phosphorylate many transcription factors such as c-Myc, p62TCF/Elk-1, c-Jun, ATF2, MEF2C, FOS and SAP-1 (Kong et al., 2001). The net result of this cascade is the altered activity of the transcription factors, which dimerise and bind to specific DNA sequences called response elements, thus promoting transcription of growth related genes. Figure 1.9 represents an example of the signal transduction molecular events, which take place upon binding of a growth factor to its receptor.

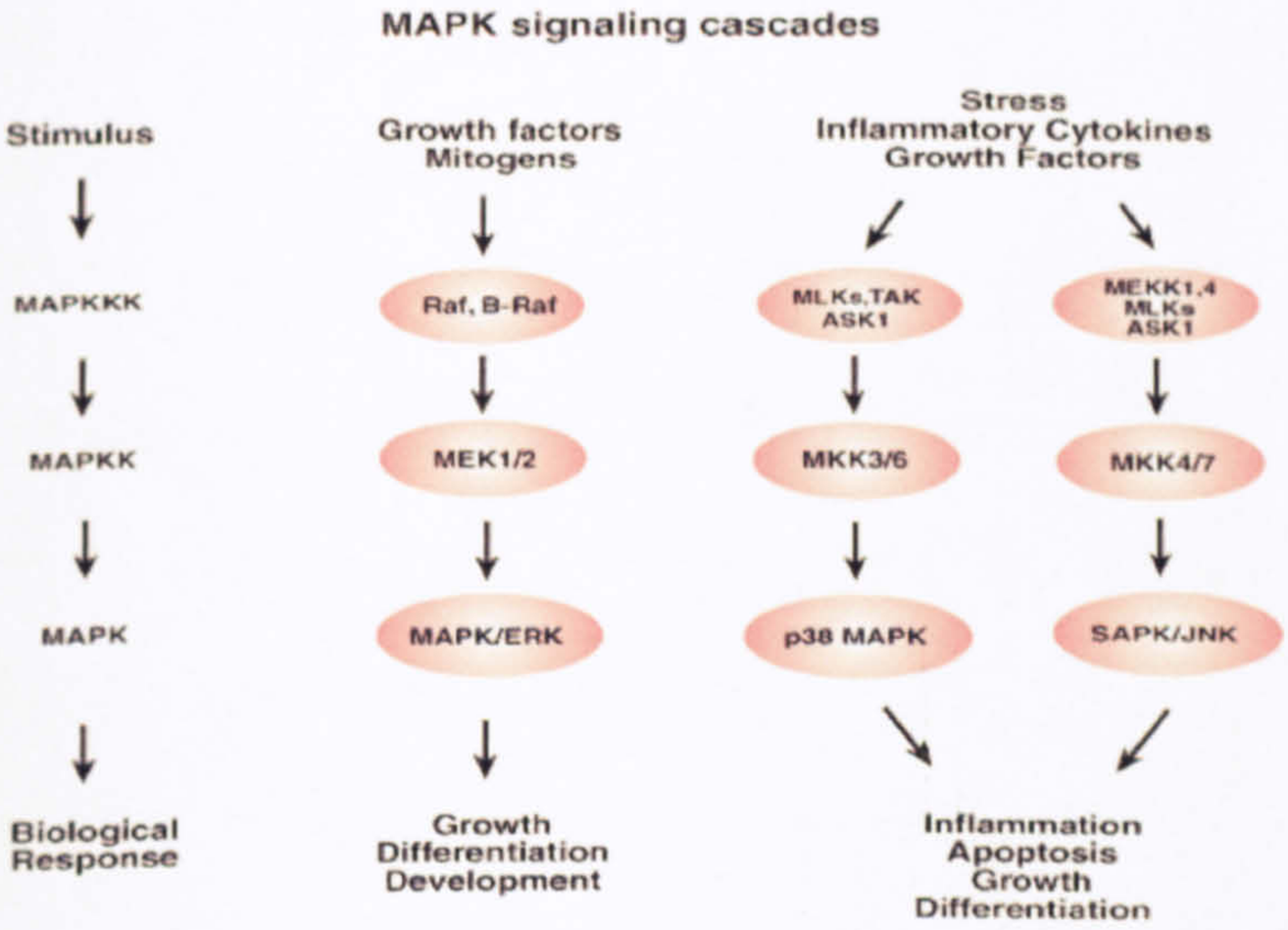


Figure 1.9. Mitogen activated protein kinase (MAPK) cascades. Signalling through the MAPK cascades leads to altered activity of target genes, including several transcription factors. The picture also shows the involvement of Ras in relaying the signal from the growth factors to Raf, MEK1/2 and ERK1/2 kinases. Adapted from Manson 2003

1.1.5.6.2 The Ras GTP binding protein

The Ras protein is a 21 kDa GTP binding protein, which relays the signal produced by the mitogenic growth factors to the MAPKs, phosphoinositols or cytoskeletal proteins. Ras (Figure 1.9) which is located on the cytoplasmic face of the cell membrane, is active when bound to GTP and inactive with GDP. The protein contains intrinsic GTPase activity, which inactivates its function. However another protein, GAP is needed for this process, which is an inhibitor of Ras activation. Ras is synthesised as a precursor with a C-terminal CAAX motif (C=cysteine, A=Aliphatic amino acid, X=variable). The last three amino acids are cleaved by proteolysis and the cysteine group is modified by

attachment of a 15-carbon isoprenyl (farnesyl) group, catalysed by the enzyme farnesyl transferase. Downstream effector, as shown in Figure 6 is Raf, a serine threonine protein kinase, which plays a crucial role in Ras activation by recruiting it to the cell membrane. Raf is the first serine/threonine kinase in the ERK pathway.

The ras oncogene is mutated in about 40% of all human cancers (reviewed in King, 2000). The mutations destroy the GTPase activity of Ras and hence it remains in the active GTP-bound form. Tumour associated mutant Ras proteins harbor single amino acid substitutions, primarily at residues 12, 13 and 61 that render Ras insensitive to GAP stimulated GTP hydrolysis (Pruitt and Der, 2001). These oncogenic mutants of Ras continue to signal in the absence of extracellular signals.

Except the MAPKs the second best characterised downstream effectors of Ras are the phosphoinositide-3-phosphate lipid kinases (PI3Ks). Activated PI3K, converts phosphatidylinositol 4,5-phosphate (PIP_2) to phosphatidylinositol 3,4,5-phosphate (PIP_3). PIP_3 levels are usually elevated in Ras-transformed cells and promote the activation of the Akt/PKB (Protein kinase B) serine threonine kinase (Pruitt and Der, 2001).

1.1.5.7 The cell cycle and the cyclin dependent kinases (CDKs)

Mitosis only represents about 5% of the cell cycle and about 40% is required for DNA synthesis. DNA synthesis and mitosis are separated by gaps, during which RNA and proteins are made. The first gap is called G_1 and its duration covers about 45% of the cell cycle. G_1 is the regulation point which ensures that the cell has adequate "machinery" for future events and for transmission of genetic information (reviewed in King, 2000). After G_1 S phase follows where DNA synthesis occurs. Once DNA has been duplicated the G_2

phase checkpoint ensures the elimination of damaged cells, which have escaped G_1 control or which have not accurately duplicated their DNA (Pines, 1999).

The key mechanism in the control of the cell cycle, is the regulation of protein phosphorylation levels. The protein kinases involved in this process are serine threonine kinases, made up of a regulatory and a catalytic subunit. The regulatory subunits are called cyclins and the catalytic subunits cyclin dependent kinases (CDKs). There are three different classes of CDKs associated with either the G_1, the S or the G_2/M phase of the cell cycle and four different classes of cyclin proteins (cyclins D, E, A and B). Mitogenic signals promote the entry of quiescent cells into the first gap phase (G_1) and initiation of DNA synthesis in the S phase (Pines, 1999). This progression into G_1 phase is cooperatively regulated by the various classes of CDKs and their CDK inhibitors (CKIs). CKIs can be divided into two major classes: INK4, so named by their ability to inhibit CDK4 which are $p16^{INK4a}$, $p17^{INK4b}$, $p18^{INK4c}$ and $p19^{INK4d}$ and Cip/Kip family of inhibitors which are $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$ (reviewed in Sherr and Roberts, 1999). INK4 inhibitors act on CDK4 and CDK6 whereas Cip/Kip inhibitors affect the action of cyclin D-,E- and A- dependent kinases (Pines, 1999).

Transcription of *cyclin D1* is regulated by growth factors and other signals through the Ras/Raf/ERK pathway. Turnover of D-type cyclins depends on the PI3K/Akt pathway, which negatively regulates the phosphorylation of cyclin D_1 on a single threonine residue, by glycogen synthase kinase-3^{β} (GSK-3^{β}) (reviewed in Sherr and Roberts, 1999). Inhibition of this signalling pathway accelerates the ubiquitin-dependent proteasomal degradation of cyclin D1 in the cytoplasm and shortens its half life to as little as 10 min. Cyclin D-dependent kinases (CDK4 and CDK6) phosphorylate Rb in mid G1 phase, after

which cyclin E-CDK2 becomes active and completes this process by phosphorylating Rb on additional sites (Sausville, 2005). Rb hyperphosphorylation in late G1 disrupts its association with various E2F members of transcription factors and this allows the transcription of various genes which are required for DNA synthesis (Sausville, 2005). Cyclin A- and B- dependent CDKs maintain Rb in a hyperphosporylated state until cells exit mitosis and Rb is returned to a hypophosphorylated state in the next G1 phase (Sausville, 2005). When overexpressed E2F can drive quiescent cells into S phase, whereas active repression by the Rb-E2F complex, as opposed to a block in E2F transcriptional activation, is required to enforce G1 arrest by certain antiproliferative signals (reviewed in Sherr and Roberts, 1999). A representation of the cell cycle is shown in Figure 1.10.

The second most important function of cyclin D-CDK4 complexes is the sequestration of Cip/Kip family CIKs, such as $p21^{Cip1}$ and $p27^{Kip1}$. In proliferating cells most $p27^{Kip1}$ is complexed to cyclin D-dependent kinases (reviewed in Sherr and Roberts, 1999), while in quiescent cells $p21^{Cip1}$ levels are low and $p27^{Kip1}$ levels are high. $p27^{Kip1}$ and and $p21^{Cip1}$ bind to higher order complexes with assembling cyclin D-dependent kinases and this relieves cyclin E-CDK2 from Cip/Kip constraint, facilitating its activation in late G1 phase (reviewed in Sherr and Roberts, 1999). This activation occurs from a single phosphorylation reaction of cyclin E-CDK2 on $p27^{Kip1}$, once the process of Cip/Kip sequestration has lowered the effective CKI level to a critical point. Once CDK2 becomes active it triggers the destruction of $p27^{Kip1}$ (Pines, 1999). This results to an irreversible commitment of the cell to enter the S phase of the cycle and thus mitogenic signals are required to a lesser extent for cell cycle progression.

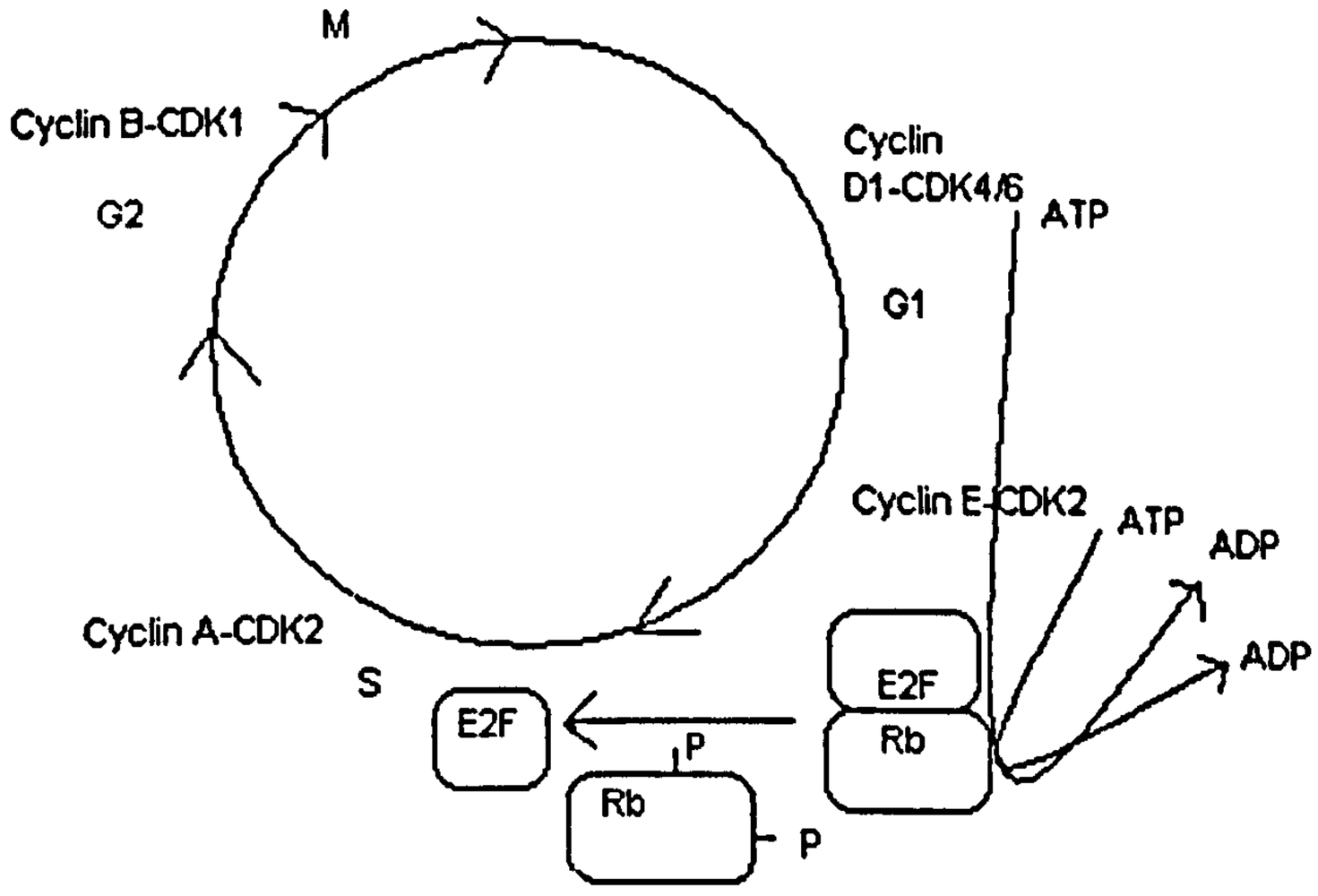


Figure 1.10. Schematic representation of the cell cycle. Cyclins and their corresponding CDK partners are shown, as well as their interaction with the Rb protein.

1.1.6 Cancer treatment

Cancer treatment requires the combination of various techniques. Cancer cells can be removed surgically or destroyed with toxic chemicals or radiation. Unfortunately it is usually very difficult to eradicate all cancer cells. Surgery can rarely remove every metastasis and chemotherapy is usually toxic to normal cells as well. If a few cancerous cells survive they can proliferate to produce a reccurence of the disease and may develop resistance against chemotherapeutic agents.

1.1.6.1 Cytotoxic chemotherapy

Chemotherapy has been the main form of drug treatment at all stages of cancer development over the last 40 years. Chemotherapeutic drugs, which were first discovered to treat cancer patients can be classified broadly into three categories, according to their mechanism of action: Alkylating agents, antimetabolites and natural products.

1.1.6.1.1 Alkylating agents

Alkylating agents have two functional groups each of which can react with a DNA base and form interstrand and intrastrand crosslinks within the DNA double helix (reviewed in King, 2000). This causes the formation of DNA adducts that disrupt DNA synthesis. Two of the most commonly used alkylating agents are cyclophosphamide and *cis*-platin (Figure 1.11). These two agents react with DNA bases and form cross-linked guanines, which block proliferation at several stages of the cell cycle. Cyclophosphamide is used in combination with methotrexate and 5-fluorouracil for the treatment of many cancers. Its side effects include sterility, hair loss and immunosuppression. *Cis*-platin forms DNA adducts at the N-2 position of guanine or N-3 position of adenine (Hurley, 2002). Intrastrand adducts distort the DNA helix, whilst interstrand links prevent strand separation so that replication is inhibited. *Cis*-platin is clinically used against testicular and ovarian cancers and it has minimal effect on the bone marrow. Its main toxicities are nausea, renal dysfunction and renal effects.

1.1.6.1.2 Antimetabolites

Antimetabolites inhibit nucleic acid synthesis with the actual mechanism depending on the compound. The most widely known are methotrexate and 5-FU (Figure 1.12).

Methotrexate is a derivative of folic acid and is a competitive inhibitor of the enzyme dihydrofolate reductase (DHFR), which is essential for purine and pyrimidine biosynthesis (reviewed in King, 2000). Folic acid (FH_2) is reduced to tetrahydrofolic acid (FH_4) by DHFR and the latter can form N5,N10 methylene tetrahydrofolic acid or N10 formyl FH_4 which are used for purine synthesis (reviewed in King, 2000). Methotrexate inhibits the reduction of folic acid to tetrahydrofolic acid and it can treat the cancer choriocarcinoma on its own, but its toxic effects affect the bone marrow (reviewed in King, 2000).

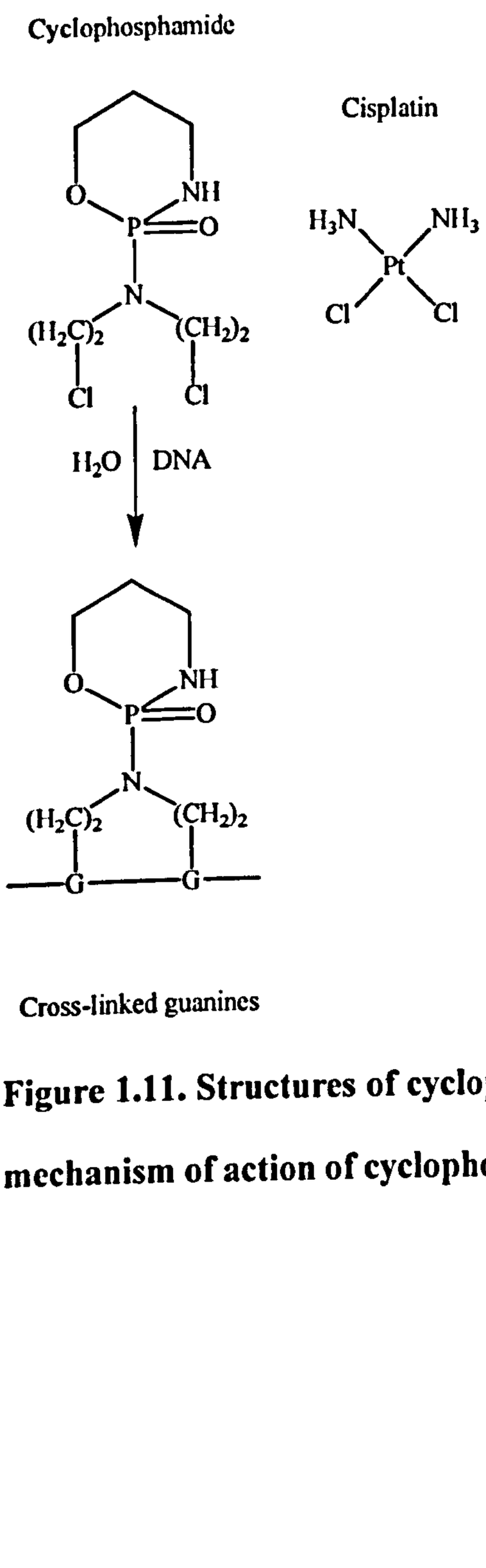
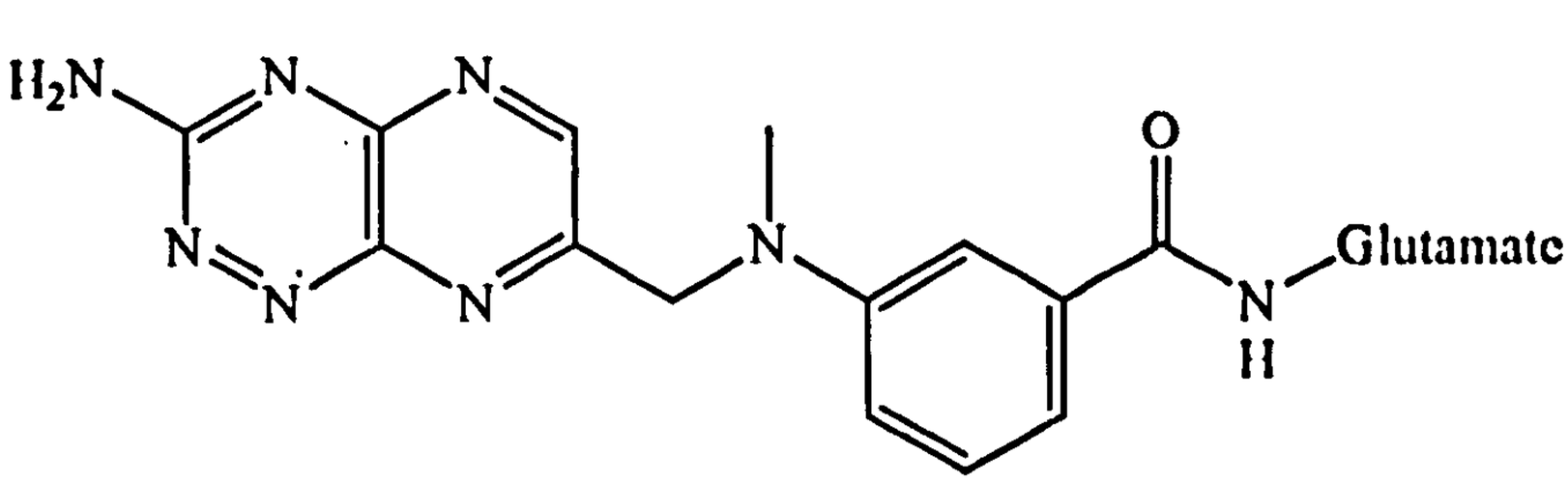
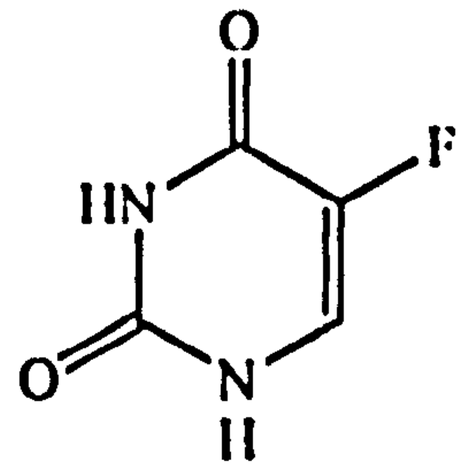


Figure 1.11. Structures of cyclophosphamide and *cis*-platin. The reaction demonstrates the mechanism of action of cyclophosphamide



Methotrexate



5-Fluorouracil

Figure 1.12. The stuctures of methotrexate and 5-FU

5-FU is a derivative of uracil that can be phosphorylated and incorporated into RNA. Additionally it produces 5-fluoro-dUMP that forms an inactive ternary complex with the N5,N10-methylene-FH_4 thereby inhibiting DNA synthesis (reviewed in King, 2000). It is used to treat breast and stomach cancers and like methotrexate shows toxicity in bone marrow cells.

1.1.6.1.3 Natural products

Doxorubicin is one of the natural products discovered to treat cancer. It acts as a non specific DNA intercalating agent and blocks the religation step of the DNA by inhibiting the enzyme topoisomerase II (Hurley, 2002). It is also likely that other mechanisms, such as direct oxidative damage to DNA might contribute to the overall efficiency of the drug (Hurley, 2002). It is used for the treatment of leukaemias and solid cancers such as breast, lung and ovary and it shows side effects on heart function (reviewed in King, 2000).

Vincristine and vinblastine are two other natural products that are used as chemotherapeutic agents. These two plant alkaloids act by preventing tubulin polymerisation. They bind to tubulin and by disrupting the mitotic spindles, they cause a

block of the cell cycle in mitosis. Taxol™ (paclitaxel) is a terpene that acts at the same locus but prevents tubulin depolymerisation (reviewed in King, 2000). These compounds have bone marrow and neural toxicity and are used to treat a broad spectrum of cancers such as leukaemia, ovarian cancer and testicular cancer.

1.1.6.1.4 Hormone related cancer therapy

In some types of cancer, such as breast and prostate, steroids are the main compounds, that drive cell proliferation, through continous stimulation of cells via specific receptors. Treatment of hormone sensitive cancers is based on the principle of depriving them of the mitogenic hormone. This can be achieved either by blocking the target cell receptor machinery or by preventing steroid synthesis.

Steroid receptor antagonists have been used to treat hormone related cancers. The most widely known anticancer compound that belongs to this class is tamoxifen. Tamoxifen is a stilbene which has been used to treat breast cancer. It antagonises binding of the hormone oestradiol to the oestrogen receptor.

Steroid synthesis can also be prevented though, by agonists of the hormones released from the hypothalamus-pituitary axis. The secreted hormone from the hypothalamus or the hormone secreted from the pituitary can be inhibited by agonists that bind very strongly to the hormone receptors so that they downregulate secretion of downstream hormones e.g. from the pituitary or the third endocrine gland. An example is LHRH superantagonists which have been used for the treatment of prostate cancer. LHRH is released from the hypothalamus and stimulates the pituitary gland for formation of LH, which in turn acts on the prostate to elicit secretion of testosterone (Figure 1.13). The

inhibitors act by desensitizing the pituitary gland causing decreased formation of testosterone by the prostate.

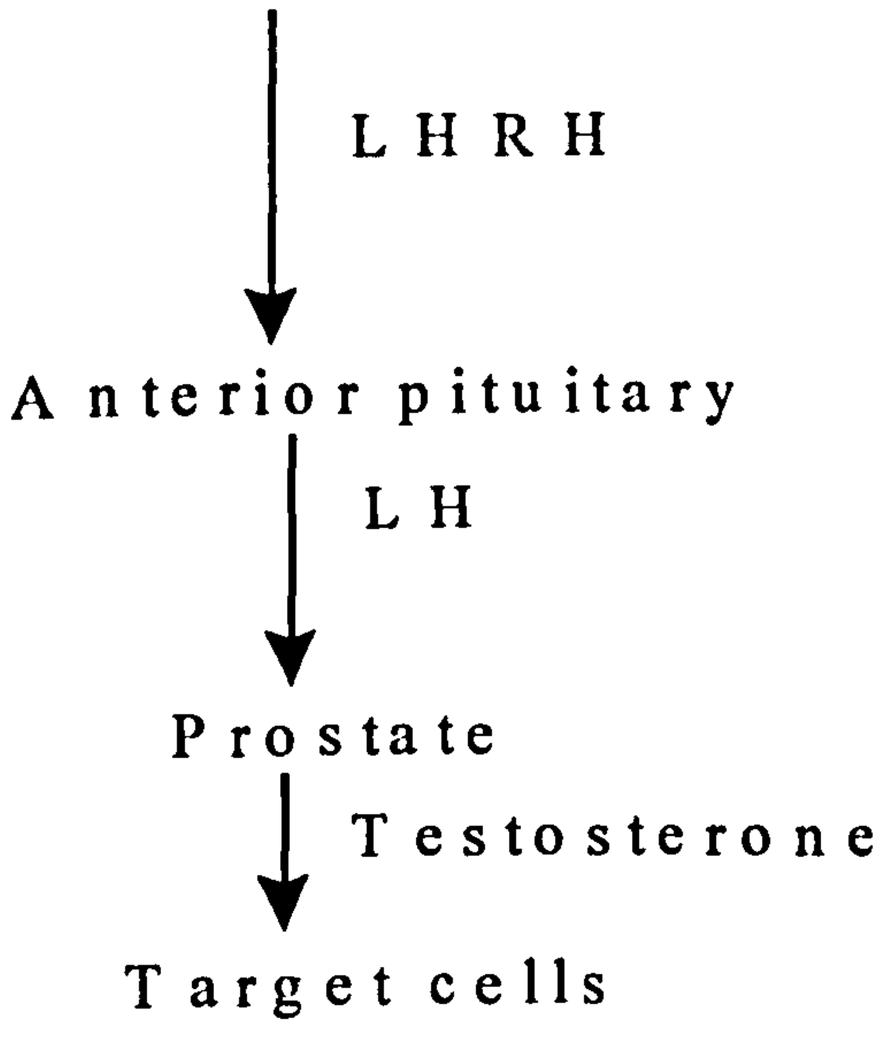


Figure 1.13 Regulation of testosterone secretion by the hypothalamus and pituitary

1.1.6.2 Chemotherapy-Molecular approaches

The main disadvantage of conventional chemotherapy is the severe side effects seen in the normal tissues. The first chemotherapeutic agents discovered were inhibiting key processes in DNA replication and cellular proliferation, as discussed earlier, thus acting on all dividing cells and lacking selectivity towards the tumour cells. In the last 10 years, targeted therapy has emerged, as an approach that would significantly reduce undesired side effects and would increase drug killing at the tumour site.

1.1.6.2.1 Tyrosine kinase inhibitors

As discussed in section 1.1.3.3, transmembrane receptor tyrosine kinases play a pivotal role in growth factor signalling, and their overexpression can lead to malignant transformation. As a result, receptor tyrosine kinases have become a favourite target for drug development.

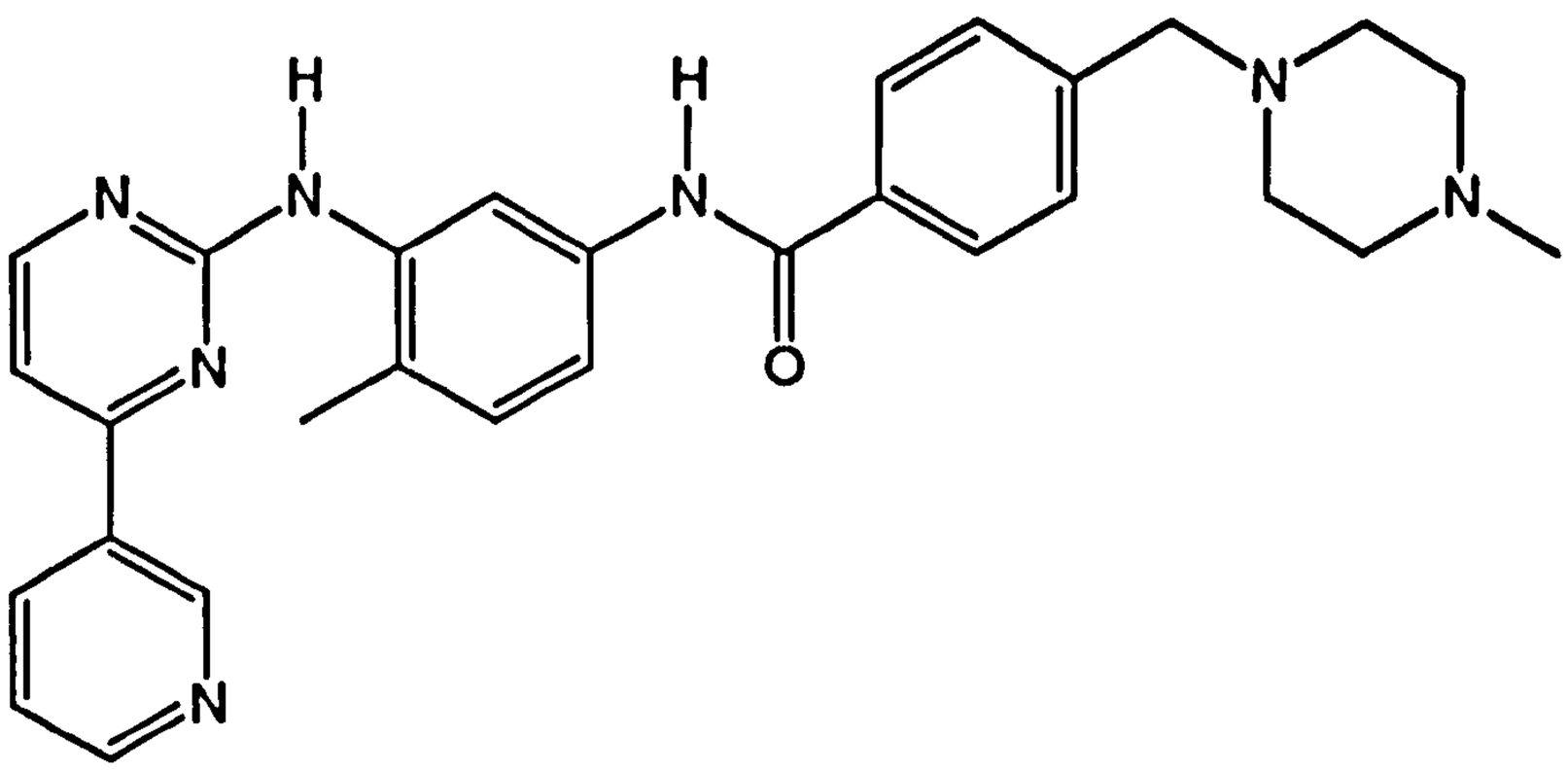
The first promising tyrosine kinase inhibitor was Imatinib mesylate (or STI571, Gleevec^{TM}) (Figure 1.13), which was developed by Novartis in 1996, and approved by the FDA in 2001 for the treatment of chronic myeloid leukaemia (CML), and unresectable or metastatic malignant gastrointestinal stromal tumours (GIST). Imatinib acts by blocking the ATP binding site of Bcr-Abl and c-Kit kinases, thus preventing phosphorylation reactions required for signal transduction that stimulate cell proliferation (Druker et al., 1996). In CML the Abelson (Abl) kinase is improperly activated by the accidental fusion of the *bcr* gene with the gene encoding the intracellular non-receptor tyrosine kinase c-Abl (Druker et al., 1996). Under normal conditions c-Abl exists in a regulated state with a very low kinase activity (Buchdunger et al., 1996), whereas in CML the fusion of Bcr to the NH_2 terminus of c-Abl results in the constitutive activation of Abl kinase activity by a mechanism that is not well understood (Nagar et al., 2002). Imatinib also inhibits platelet derived growth factor receptor (PDGFR) and is currently undergoing phase I/II clinical trials for the treatment of recurrent malignant gliomas and phase II to treat childhood soft tissue sarcoma bone cancer (Guillemard and Saragovi, 2004). Imatinib is one of the most promising anticancer drugs ever developed, because it inhibits several tyrosine kinases and further drug development has concentrated on the

inhibition of similar tyrosine kinase targets, such as the EGFR and VEGFR family of receptors.

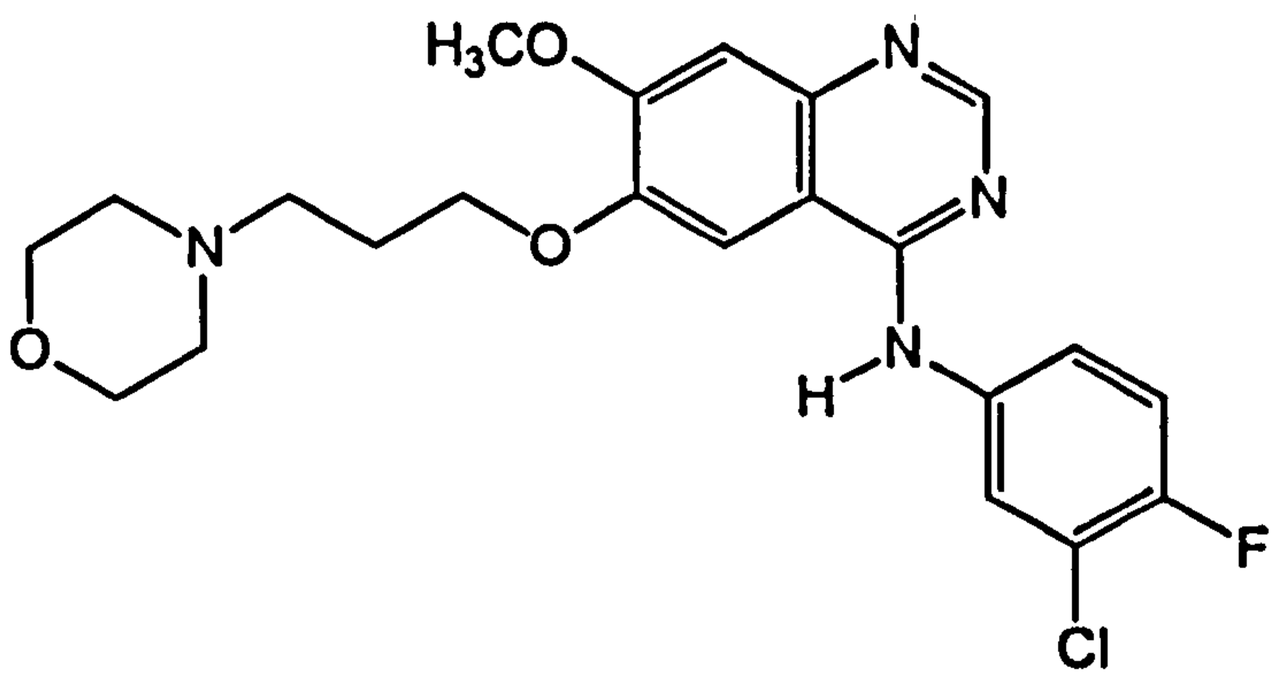
1.1.6.2.1.1 EGFR directed strategies

The quinazolines Gefitinib (ZD1839) and Erlotinib (OSI774) (Figure 1.13), which are the most advanced in clinical development, are competitive inhibitors at the tyrosine kinase ATP binding site of the EGFR. Gefitinib has been licensed for use in relapsed advanced NSCLC (Non small cell lung carcinoma) in Japan, Australia and USA under the name of Iressa and is being studied in phase II trials along with Erlotinib (Tarceva) in patients with advanced squamus cell carcinoma of the head and neck (SCCHN) (Ranson, 2004). Response rates of approximately 5-10% have been reported with up to a third of patients showing stable disease (Ranson, 2004). Erlotinib is also undergoing phase I to III clinical trials for the treatment of pancreatic cancers as well as the metastatic carcinoma of the endometrium alone or in combination with standard chemotherapy. An advantage of Gefitinib is that the desired therapeutic effect can be achieved at a dose below the MTD, (maximum tolerated dose) as demonstrated in clinical trials for relapsed NSCLC. Furthermore this agent has been shown to potentiate the effect of current cytotoxic agents, such as paclitaxel or docetaxel, in combination treatment against A431, LX-1, SK-LC-16, TSU-PR1 and PC-3 tumour xenografts (Sirotnak et al., 2000). Co-administration of Gefitinib and the taxanes paclitaxel or docetaxel resulted in partial or complete regression of the above tumour xenografts, whereas only growth inhibition was observed when the taxanes were administered alone (Sirotnak et al., 2000). Potentiation

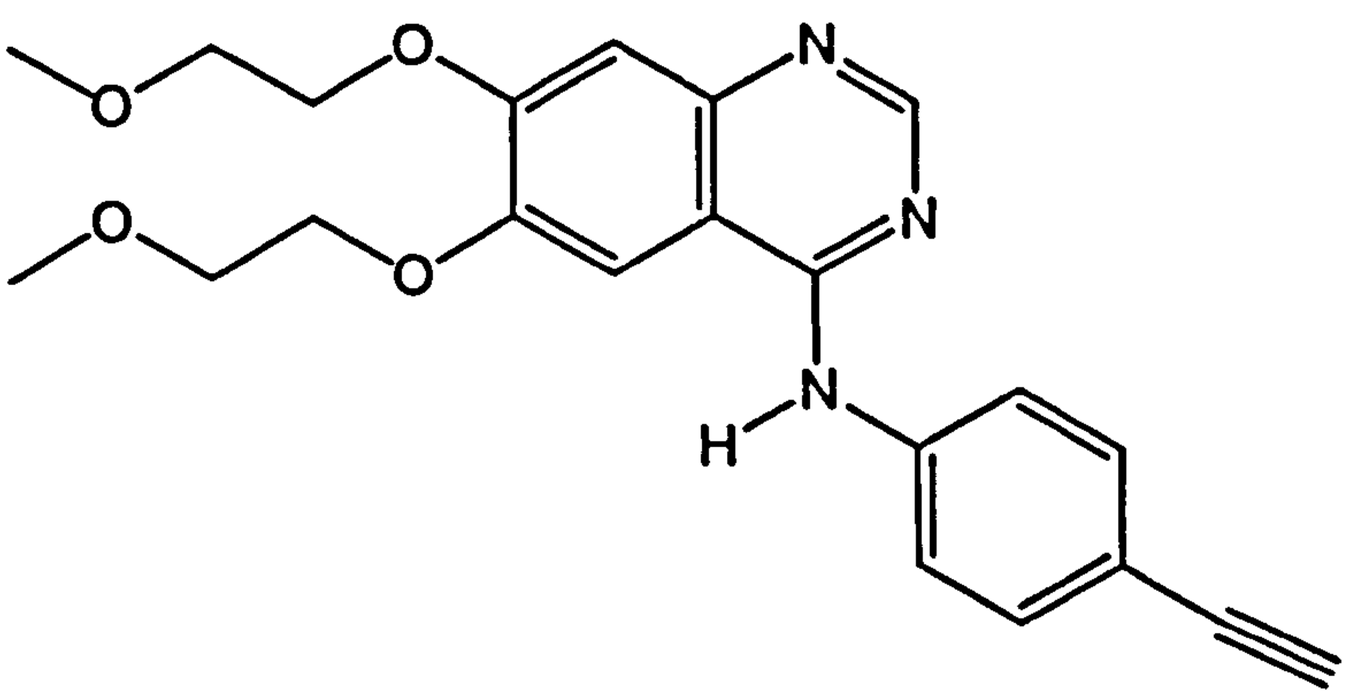
of cytotoxic treatment in the above preclinical models did not require high levels of EGFR expression in the target tumours, as demonstrated by immunohistochemical and RT-PCR studies (Sirotnak et al., 2000).



Imatinib
(Gleevec^{TM})



Gefitinib
(Iressa^{TM})



Erlotinib
(Tarceva^{TM})

Figure 1.14. Structures of the most promising recently developed tyrosine kinase inhibitors

Such preclinical observations raised the hope, that the addition of Gefitinib to cytotoxic chemotherapy would result in improved efficacy. However, two randomised clinical phase III trials, involving 2000 patients with stage III/IV NSCLC, one with carboplatin/paclitaxel and the other with *cis*platin/gemcitabine, did not demonstrate that the addition of Gefitinib improves the response rates, time to progression or survival compared to combination chemotherapy alone (Ranson, 2004).

CI1033, PKI166, EKB569 and GW572016, which produce inhibition of multiple EGFR family members, are in early phase clinical development (Ranson, 2004). CI1033 is a potent kinase inhibitor of all members of the erbB receptor family, under development by Pfizer, which produces irreversible inhibition of EGFR kinase with an IC_{50} in the low nanomolar range and antitumour activity in EGFR and erbB2 dependent preclinical models (Ranson, 2004). Phase II trials are now in progress. PKI166 is a reversible dual EGFR and erbB2 tyrosine kinase inhibitor, under clinical development by Novartis which entered clinical trials in 1999. PKI166, except inhibiting epithelial tumor growth and angiogenesis, has been shown to potentiate the activity of gemcitabine in preclinical models of pancreatic cancer (Ranson, 2004). GW572016 is a reversible dual erbB2 and EGFR tyrosine kinase inhibitor under development by GlaxoSmithKline, which has passed phase I in NSCLC and EKB569 is an irreversible dual inhibitor of EGFR and HER2 tyrosine kinases, which is currently under clinical phase II trials (Ranson, 2004).

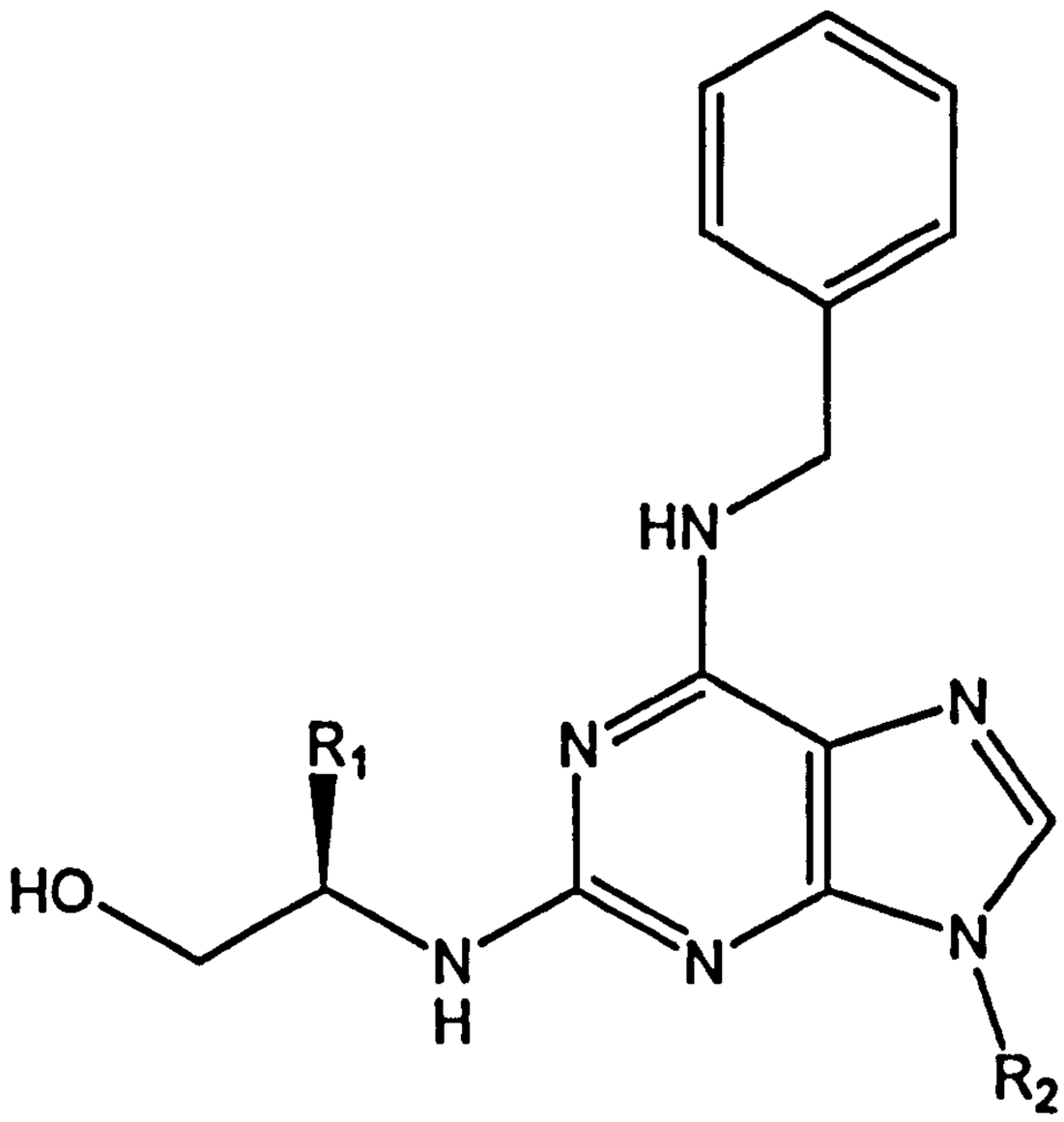
1.1.6.2.1.2 VEGFR inhibitors

Much work has been done on the inhibition of the VEGF receptor. IMCIC11 is a mAb that targets VEFGR2 and was evaluated in phase I for the treatment of colorectal cancer (Guillemard and Saragovi, 2004). Beracizumab is a recombinant humanised mAb against VEGF, currently in phase III for the treatment of renal cell carcinoma, phase II for metastatic prostate cancer, relapsed or refractory multiple myeloma, malignant mesothelioma, breast cancer and non-Hodgins lymphoma (Guillemard and Saragovi, 2004). Such antibodies target the extracellular domain of the VEGFR. Targeting the intracellular domain of the VEGFR has been a popular approach with over 40 antiangiogenic drugs currently undergoing evaluation in phase I, II or III clinical trials. The small molecule inhibitor SU5416 (Semaxanib) inhibits VEGFR2 and is in phase II for the treatment of a variety of solid tumors, such as Kaposi sarcoma, mon small cell lung and breast cancer and in phase III for metastatic colorectal cancer (Guillemard and Saragovi, 2004). The small molecules PTK787/ZK22584 and CGP4125 inhibit VEGFR2 and are in phase I/II clinical trials for the treatment of solid tumors (Guillemard and Saragovi, 2004). ZD6474 is an orally bioavailable VEGFR and VEGFR2 receptor tyrosine kinase inhibitor, which has additional activity against EGFR and fms-like tyrosine kinase 4 (VEGFR3) (Ranson, 2004). It is in early phase II clinical trials with broad spectrum anticancer efficacy.

1.1.6.2.2 CDK inhibitors

Tumour cells possess faulty checkpoints and can proliferate despite a compromised genome. Very often the mechanisms by which transformed cells override checkpoints are closely related to CDK function. For this reason restoration of cell cycle control through pharmacological inhibition of CDKs has been actively pursued over the last 10 years as a new strategy to treat cancer.

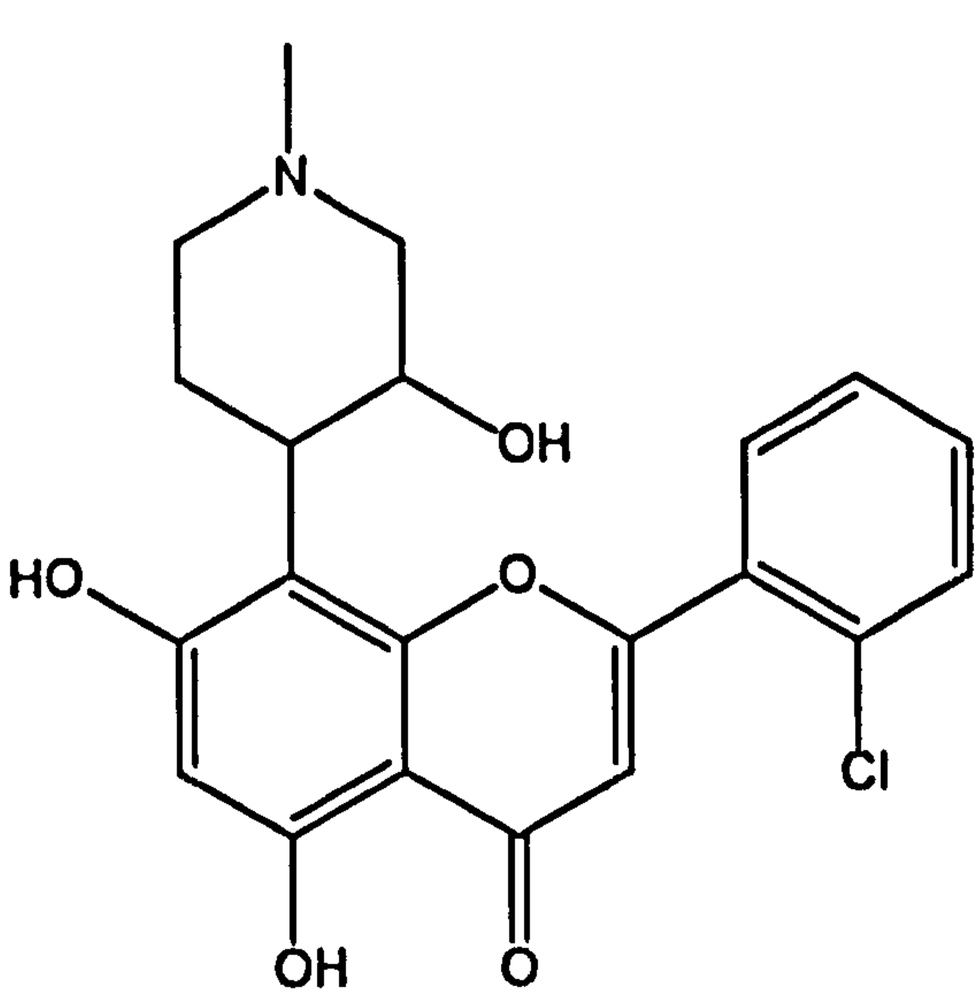
Initial attempts to design CDK inhibitors were based on substituted purines/adenines which will antagonise the binding of ATP (Kim, 1998). Some examples are shown in Figure 1.13. Flavopyridol (Figure 1.16) was the first pharmacologic CDK inhibitor. It was originally discovered as an EGF-receptor tyrosine kinase inhibitor but it was later found to be 100-fold more potent as a CDK inhibitor. The mechanism of action of flavopyridol has not been fully understood and extensive structure activity relationships around flavonoid compounds have been established. Flavopyridol has been demonstrated to inhibit CDKs 1,2,4,6 and 7 as well as the CDK9/cyclin T complex (reviewed in Blagden and de Bono, 2004). It also targets protein kinase A and C. Rationally designed flavopyridol mimics such as the thio and oxo flavopyridol analogues appear to have good CDK selectivity and potency particularly for CDK1, but possess only modest cellular activity.



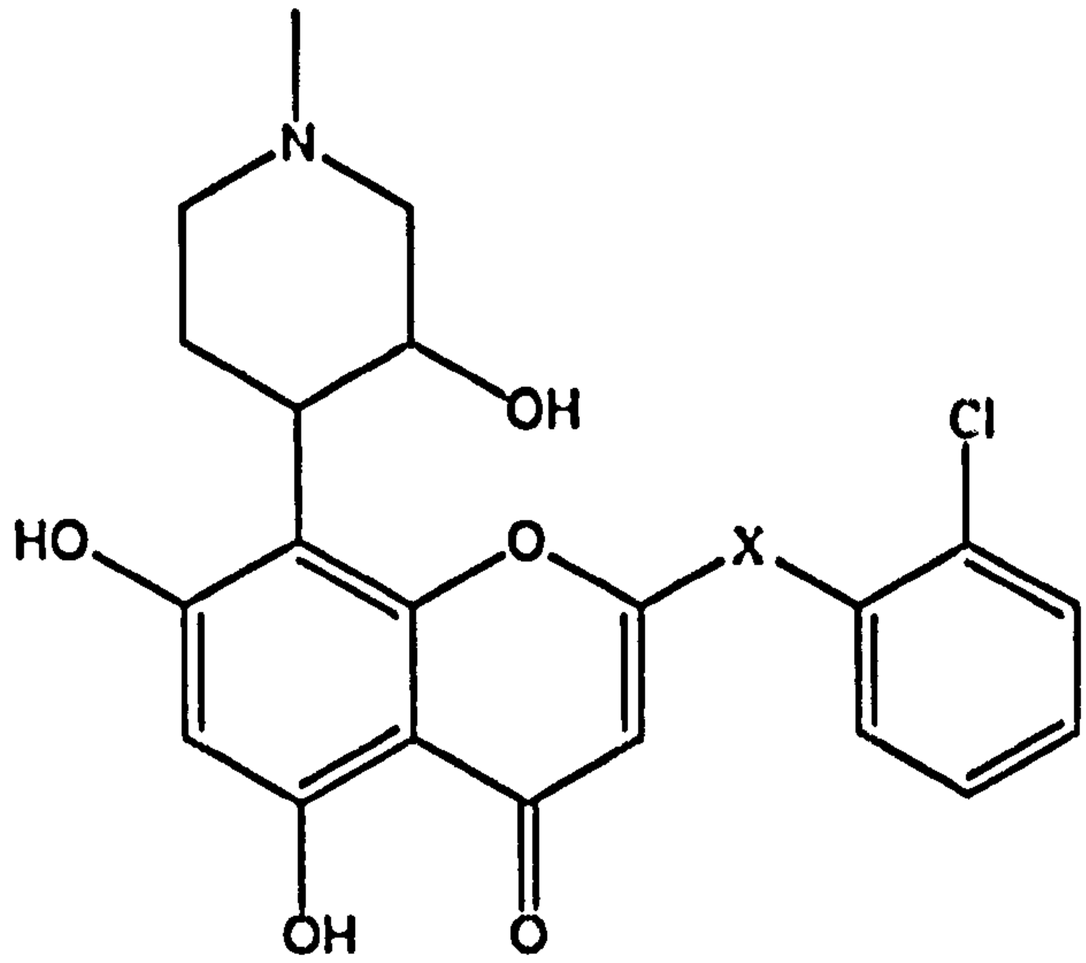
Olomoucine R_1=H R_2=Me
Roscovitine R_1=Et R_2=iPr

Figure 1.15. CDK inhibitors with purine based structures

To date no compound has been identified that has individual CDK selectivity and perhaps this is unlikely to happen due to the high homology between individual CDKs, particularly within the ATP binding pocket. Nevertheless such highly selective inhibitors would be very useful as therapeutic agents and for cellular pharmacology studies.



Flavopyridol



X=S Thioflavopyridol
X=O Oxoflavopyridol

Figure 1.16 Flavopyridol and structurally related CDK inhibitors

1.1.6.2.3 HDAC inhibitors

Histones are proteins, which are involved in the formation of the chromatin structure of DNA. Acetylation and deacetylation of histone proteins are known to have profound influences on the regulation of gene expression and on other processes acting on genomic DNA in chromatin. Acetylation occurs at lysine residues of histones and is regulated by the opposite activities of histone acetyltransferases (HATs) and histone deacetylase enzymes (HDACs).

Recent studies show that inhibition of HDACs elicits anticancer effects in several tumor cells by inhibition of cell growth and induction of cell differentiation. As a result there has been great interest for the identification and synthesis of novel HDAC inhibitors as anticancer drugs. Natural products, such as trichostatin A (TSA) and the cyclic

tetrapeptides apicidin and trapoxin as well as synthetic inhibitors such as suberanilo hydroxamic acid (SAHA) have been studied in cancer cell lines and in tumor animal models as new approaches for cancer chemotherapy (Wang et al., 2005).

A major task of clinical cancer research focuses on how to kill cancer cells selectively. Further scientific research on cell biology and related areas has advanced the understanding of how cancers initiate and develop and aims to exploit shuttle differences between normal and cancer cells, which could eventually lead to the design and synthesis of novel selective chemotherapeutic drug.

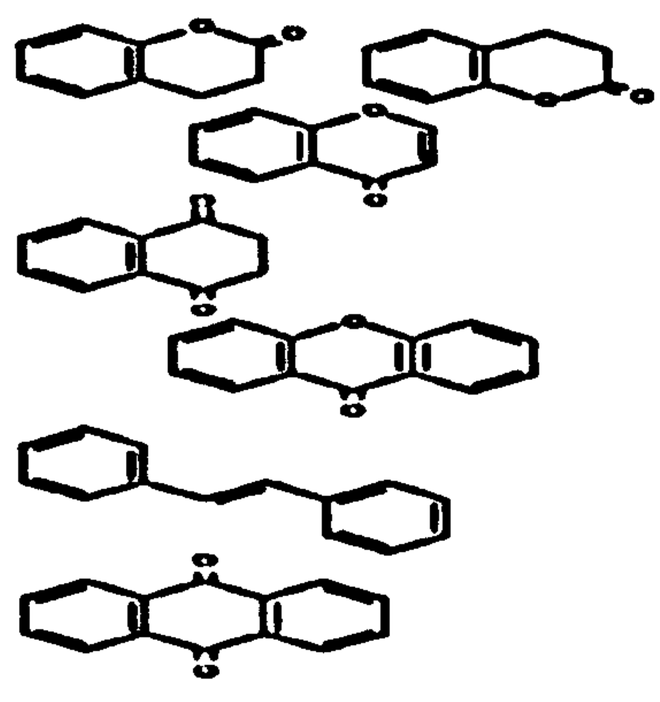
Finally, there is also a focus towards chemoprevention instead of chemotherapy. Notably natural compounds which are found in the diet have been shown to exert beneficial effects against cancer development. Plant foods, vegetables and fruits contain a wide variety of phytochemicals, which may reduce cancer susceptibility, as shown by the inverse association between their consumption and cancer risk, in epidimiological studies (Messina et al., 1998). Some of these chemopreventative agents are promising, because they are generally non toxic and able to interfere with the steps of carcinogenesis.

Cancer can be prevented by agents that decrease the activation of chemicals to their highly reactive state, increase the detoxification of chemical carcinogens or prevent their binding to their cellular targets. Amongst the various natural products present in the diet, the class that has been more extensively studied for its cancer preventative properties, is the flavonoids. Flavonoids can prevent cancer through various mechanisms of action such as antioxidant, antioestrogenic, antimutagenic, antiinflammatory activity, induction of detoxification enzymes etc.

1.2.1 Flavonoids overview

A phenolic molecule is composed of a benzene ring and a hydroxyl group attached to it. It is often characteristic of a plant species or even a particular organ tissue of that plant and plays an important structural role in the plant cell wall. Polyphenol is the name used to encompass many different forms of phenolic compounds. The actual definition comes from the Greek "πολύ" which means a lot, but the term was introduced some years ago to replace the term vegetable tannin and used the following definition: Water soluble phenolic compounds having molecular weights between 500 and 3000 and besides giving the usual phenolic reactions, such as ester formation and oxidation, they have special properties such as the ability to precipitate alkaloids, gelatin and other proteins. However, subsequent use of the term has broadened to include lower molecular weight compounds so it now covers natural products with more than one phenolic group. The main classes of polyphenols are defined according to their carbon skeleton (Table 1.3), but the most commonly found are the flavonoids, which are the most abundant polyphenols in our diet.

Table 1.3 Chemical structure and basic skeleton of some classes of polyphenols

Coumarins, isocoumarins	C_6-C_3	
Chromones	C_6-C_3	
Naphthoquinones	C_6-C_4	
Xanthones	C_6-C_1-C_6	
Stilbenes	C_6-C_2-C_6	
Anthraquinones	C_6-C_2-C_6	

Flavonoids (Figure 1.17 and 1.18) are involved in several biologically important mechanisms in plants, such as pigmentation, nitrogen fixation, repair of injury, protection against pathogens and defence against UV light (Dangles et al., 2000).

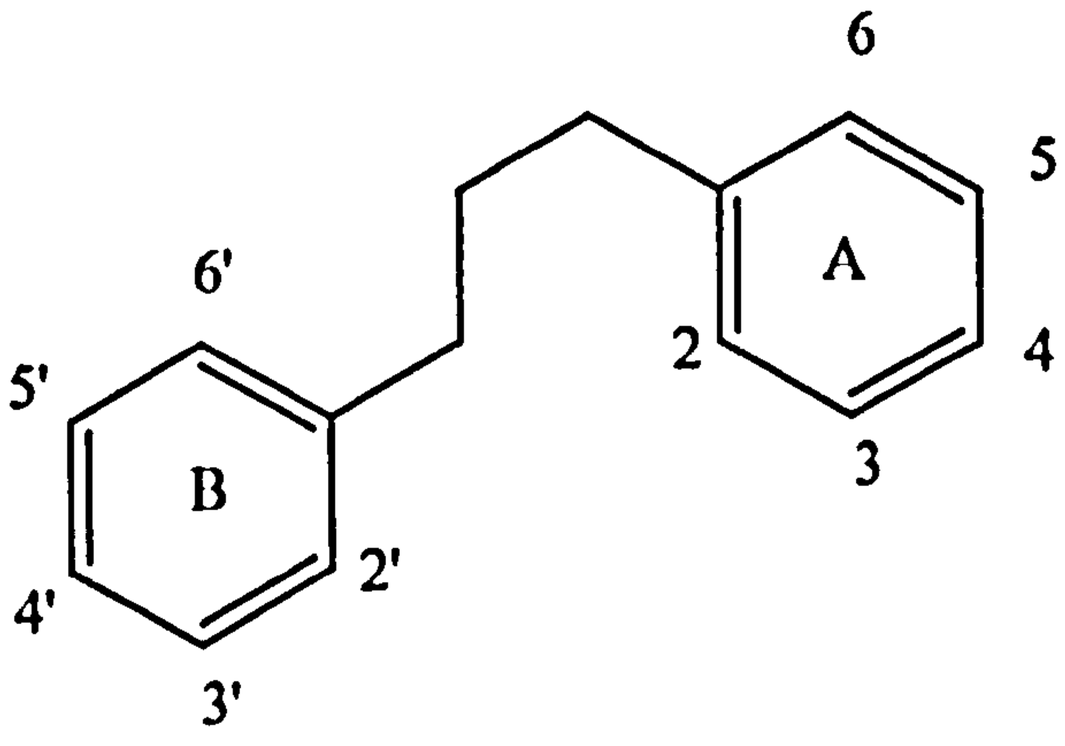


Figure 1.17 Basic flavonoid structure, containing two benzene rings joined by a linear three carbon chain

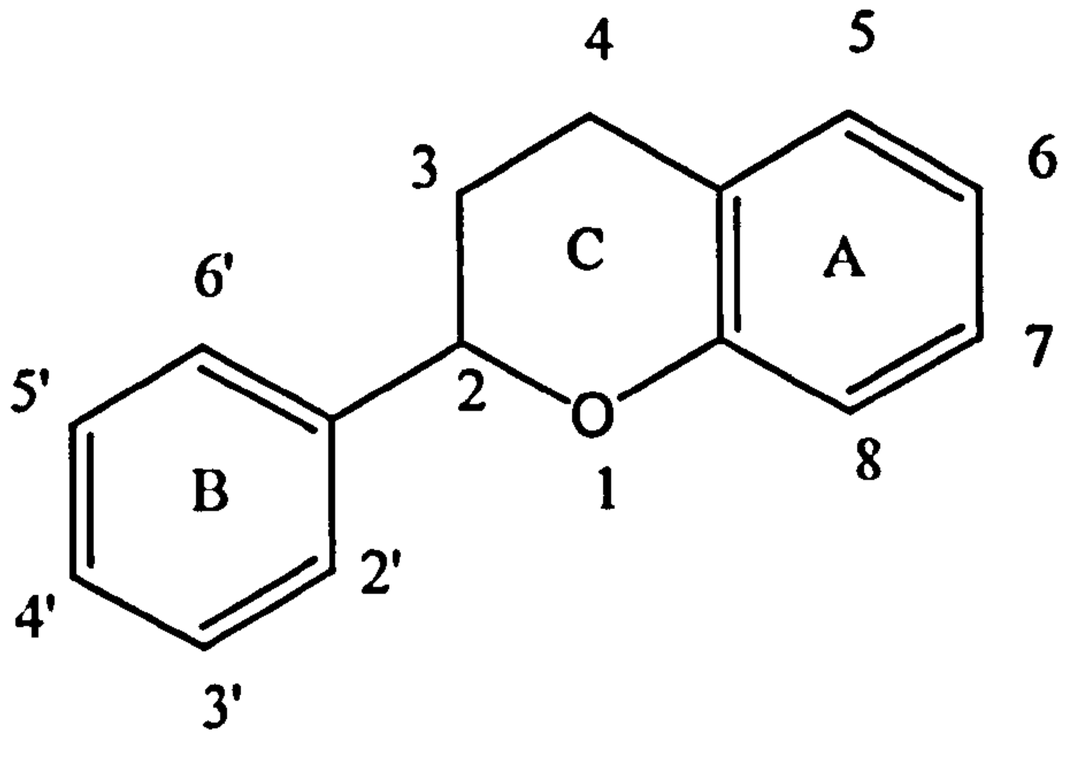


Figure 1.18 The most common form presents with a third carbon ring (C ring) between rings A and B

Over 4000 flavonoids are known. They are generally found as glycosides, which are composed of the phenolic structure, termed the aglycone and a sugar moiety termed the glycone (Figure 1.19). Glycosides are generally water soluble and are stored in the vacuoles in the plant cell. Fruits are generally richer sources of flavonoids than vegetables. Beverages such as red wine, coffee, tea and fruit juices are rich sources of flavonoids. Moreover, beer and orange juice provide a limited content of flavonoids (orange juice is more rich in ascorbate). Some trends regarding the main sources of polyphenols and flavonoids consumed can be deduced from Table 1.4.

Table 1.4 Polyphenol content of commonly consumed foodstuffs. The number of different polyphenols is shown in various quantities of different foodstuffs. Adapted from Scalbert and Williamson, 2000

Foodstuff	Phenolic acids	Flavanols	Proanthocyanidins	Anthocyanins	Catechin monomers	Reference
Potato, 200 g	28					Vinson et al 1998
Tomato, 100 g	8	1				Crozier et al 1997
Lettuce, 100 g	8	1				Vinson et al 1998
Apple, 200 g	11	7	200		21	Hertog et al 1992
Cherry, 50 g	37	1	35	200	3	Clifford 1999
Dark chocolate, 20 g			86		16	Adamson et al 1999
Red wine, 125 mL	12	2	45	4	34	Silva et al 1992

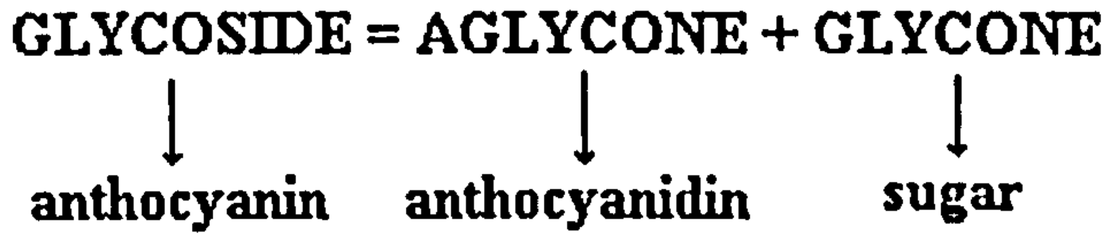
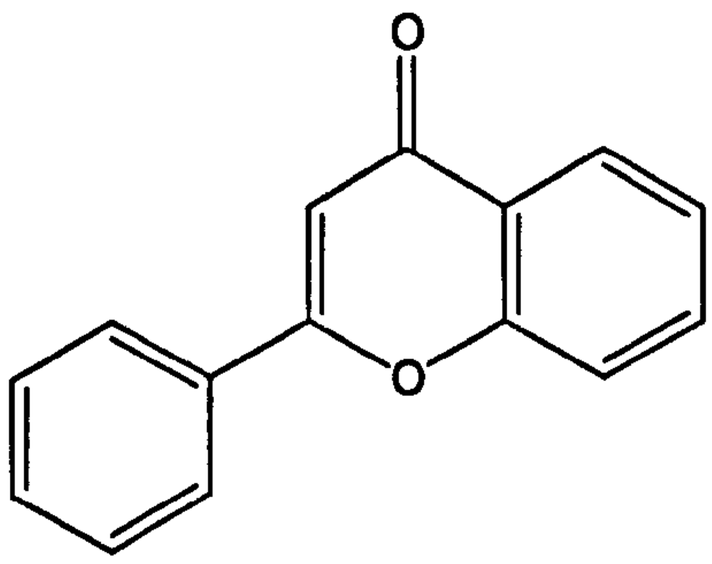
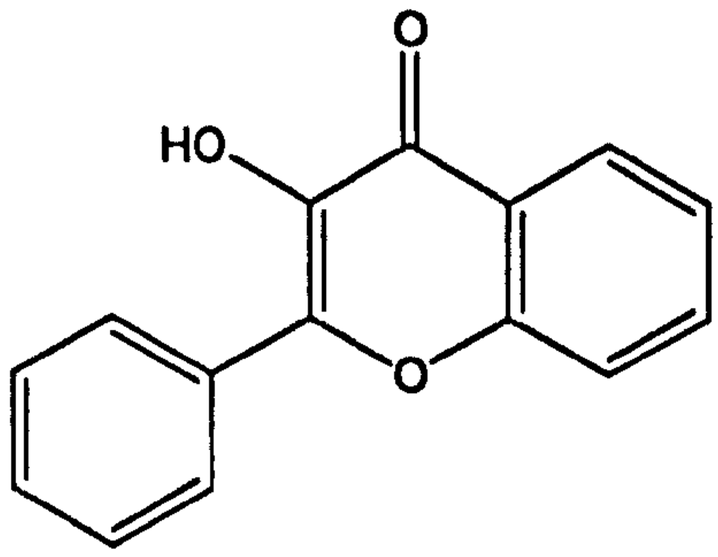


Figure 1.19 The different components of a flavonoid glycoside. Anthocyanidin is an extended conjugation made up of the aglycone of the glycoside anthocyanins. Next to chlorophyll, anthocyanins are the most important group of plant pigments visible to the human eye

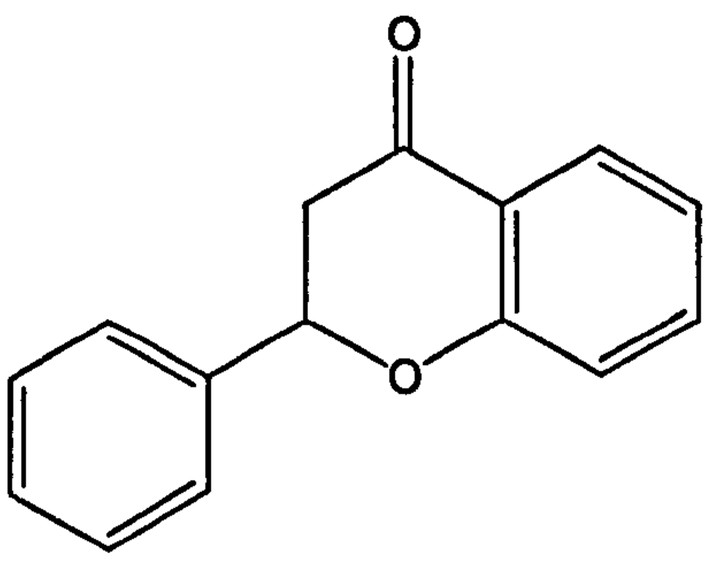
A great number of plant medicines contain flavonoids, which have been reported by many authors to have antibacterial, antithrombotic and vasodilatory actions (Halliwell and Gutteridge, 1998). There is a very great structural diversity of flavonoids, which is based on the following components: Different types of aglycone that differentiate the flavonoid subfamilies (Figure 1.20) and substitution of the aglycone by hydroxyl and methoxyl groups. The above structural components result in the great variety and the various classes of flavonoids: flavonols, flavanones, flavones, anthocyanins and isoflavonoids (Figure 1.20 and Table 1.5).



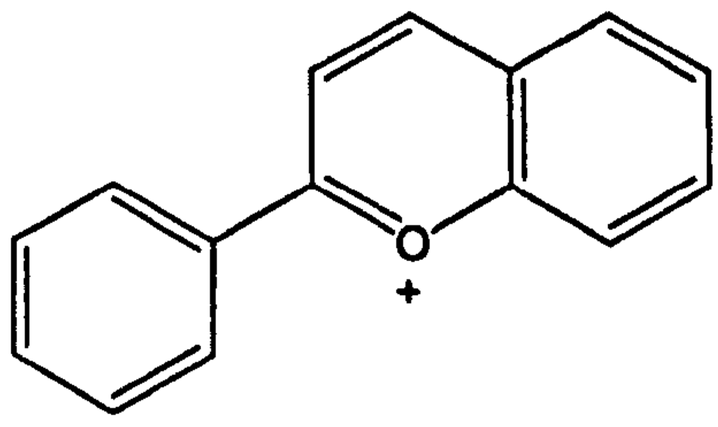
Flavone



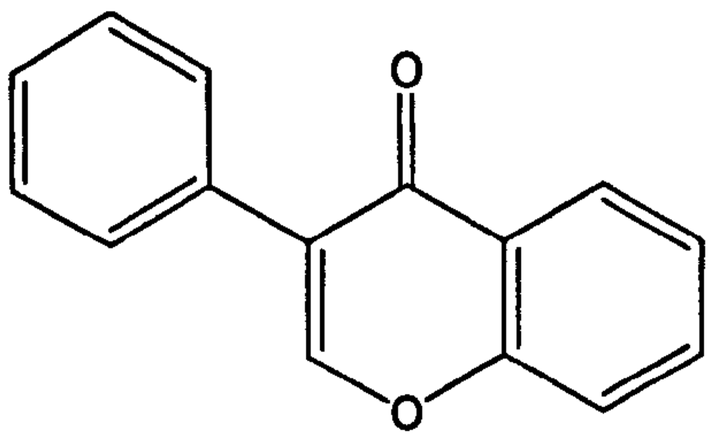
Flavonol



Flavanone



Anthocyanidin



Isoflavone

Figure 1.20 Core structures of the various subclasses of flavonoids. Further variety is caused by varying substitution patterns notably on the A and B ring

Table 1.5 Six different classes of flavonoids, including class members and plant sources

Flavonoid Class	Important Class Members	Plant Sources
Flavanols	Catechins	Green tea, grape seeds, pine bark
Flavones and flavonols	Quercetin, kaempferol	Apples, green tea, ginkgo leaves, grape skins, milk thistle fruits
Proanthocyanidins	Oligomeric catechins	Pine bark, grape seeds, leaves of bilberry, birch, ginkgo biloba
Flavanones	Hesperidin, naringin	Citrus peels
Anthocyanins, anthocyanidins and anthocyanosides	Cyanidin, delphinidin, malvidin, petunidin	Red and black grapes, red wine, bilberries
Isoflavones	Genistein, daidzein	Soy beans

1.2.2 Bioavalaibility and metabolism of flavonoids

The structural diversity of polyphenols makes the estimation of their content in food difficult. Also food fractionation may result in a loss or enrichment of some phenolic compounds (Scalbert and Williamson, 2000) . It is extremely difficult to estimate the average daily intake of polyphenols, because of structural diversity and variation of content in particular foodstuffs. However according to published results (Kuhnau, 1976), the daily Western intake of mixed flavonoids have been estimated to be in the range of 0.5 to 1 g, although recent studies have reported that the actual daily intake is lower (Hertog et al., 1993). The most abundant flavonoids in the diet are flavonols, flavanols, anthocyanins and their oxidation products. Other polyphenols, which account for one third of the total intake, are phenolic acids. The maximun concentration of flavonoids in plasma rarely exceeds 1 μM, after the consumption of 10-100 mg of a simple phenolic compound (Scalbert and Williamson, 2000).

The beneficial effects and properties of the polyphenols depend on their bioavailability and most evidence on this concept has been obtained by measuring their concentration in plasma and urine after the ingestion of either pure compounds or foodstuffs, which contain a portion of the compound of interest. There are few bioavailability studies in humans and they show that the quantities of polyphenols found intact in urine vary from one phenolic compound to another. Moreover a large part of the polyphenols ingested is not found in urine, implying an absorption and excretion process in the bile or metabolism by the colonic microflora or the body tissues (Figure 1.21). There are isolated reports in the literature (Donovan et al., 1999, Manach et al., 1998) regarding the intestinal absorption of flavonoids in humans.

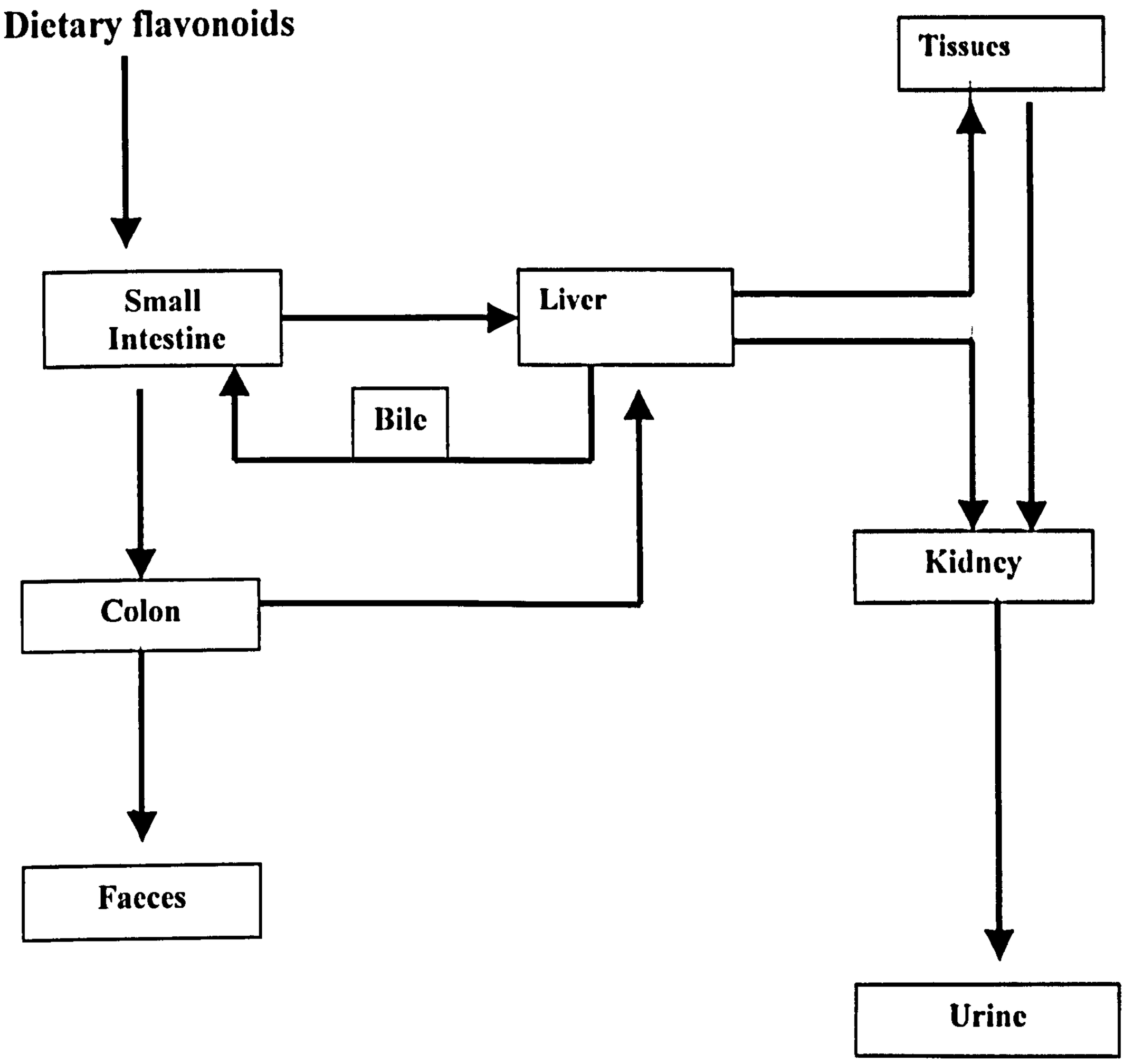


Figure 1.21 Possible routes for consumed polyphenols in humans. Adapted from Scalbert and Williamson

Table 1.6 gives an account for the content of polyphenols in humans in terms of their concentration in plasma and their excretion in urine. The maximum concentrations are often reached one or two hours after ingestion (Hollman et al., 1992).

Table 1.6 Bioavailability in humans of polyphenols consumed alone or in foods. Adapted from Scalbert and Williamson

Polyphenol	Source	Max concentration in plasma (μM)	Excretion in urine (%)	Reference
Quercetin	Onion	0.74	1.39	Hollman et al 1997
Quercetin	Apple	0.30	0.44	Hollman et al 1997
Quercetin	Apple juice		0.5	Young et al 1999
4-*O*-glucoside Quercetin	Pure compound	3.2		Hollman et al 1999
Catechin	Red wine	0.072		Donovan et al 1999
Catechin	Pure compound	2.0	0.45	Balant et al 1979
Anthocyanins	Red wine		1.0-6.7	Lapidot et al 1998

As already mentioned before certain classes of polyphenols, such as flavonols and anthocyanidins (Figure 1.3) are usually glycosylated, with the linked sugar being glucose (glucoside) or galactose (galactoside), rhamnose (rhamnoside), arabinose (arabinoside), xylose, glucuronic acid or other sugars (glycoside). Removal of the hydrophillic moiety will usually be necessary for passive diffusion across the small intestine to occur and therefore the first step of metabolism should be removal of the sugar by enzymes

(glycosidases), which occur in the food itself or in the cells of the GI mucosa. Non-enzymatic cleavage of the sugar moiety does not occur and the absorption of polyphenols is controlled by enzyme specificity with human cells expressing β-glucosidases in a tissue specific pattern and colon microflora α-rhamnosidases breaking attached rhamnose, being present in rat tissues.

It is important to note that the metabolism of polyphenols does not end with the hydrolysis of the compound to its free aglycone but there is a number of deconjugation and reconjugation reactions involved, similar to those found in the pathways followed in drug metabolism, probably occurring in the liver (Scalbert and Williamson, 2000). Therefore there is a number of enzymes involved in the polyphenol metabolism, besides glucosidases such as catechol-*O*-methyl transferase and UDP glucuronyl transferase involved in conjugation reactions i.e. methylation and glucuronidation. The metabolism is continued by the gut microflora with phenolic compounds carried to the colon, whereas these phenolics that are processed in the liver and excreted in the bile or directly from the enterocyte back to the small intestine, reach the colon as a glucuronide conjugate (Scalbert and Williamson, 2000). Colonic microflora catalyse the breakdown of the polyphenols to more stable compounds, such as phenolic acids.

1.2.3 Anticancer properties of flavonoids

1.2.3.1 Epidemiology

Many dietary factors have been associated with cancer prevention. Epidemiological studies have been consistenly shown an inverse association between consumption of vegetables and fruits and cancer risk. Plant foods contain a variety of flavonoids and isoflavonoids, which can reduce susceptibility to cancer.

One of the the most widely known epidimiological studies is the Finland study (Hertog et al., 1993). A large cohort was followed in the beginning of 1967 for 24 years and a report was published by Knekt in 1997. The incidence of cancer at all sites was inversely associated with flavonoid intake, with the protection being greatest in individuals who were under 50 years of age. The intake involved predominatly compounds such as myricetin, quercetin, luteolin and kaempferol. Another study conducted in Spain in 1998 (Garcia-Closas et al., 1998) accessed the intake of these four flavonoids in relation to lung cancer risk in Spanish women and weak associations were observed between least susceptibility to lung cancer and flavonoid intake. Similar studies in China and Japan have indicated the inverse association with soy food consumption and cancer risk, which is linked to the isoflavone genistein. Isoflavonoids are much more narrowly distributed in foods with soybeans being the primary human food that is rich in these compounds. This has led to the assumption that the lower occurrence of cancer in South East Asia, where soy-based foods are the primary food source, is caused by isoflavonoid intake. These preliminary investigations have led to the need for more extensive and conclusive studies to identify the mechanism of action of flavonoids. Prostate cancer rates were least among individuals who consumed diets abundant in rice and tofu and low in men who consumed

diets rich in seaweed (Birt et al., 2001) . There is also evidence that dietary soybeans exert a protective effect against breast cancer in South East Asia (W u et al., 1998).

1.2.3.2 Antioxidative ability

Flavonoids and isoflavonoids might protect against cancer by preventing oxidative damage of DNA which is likely to be an important cause of mutation. They serve as derivatives of conjugated ring structures and hydroxyl groups that have the potential to scavenge the superoxide anion, lipid peroxy radicals and stabilise free radicals involved in oxidative processes through hydrogenation reactions. Flavonoids can also chelate metal ions, which can give rise to hydroxide anion and alkoxy radicals via the Fenton reaction. The ability of these compounds to chelate metal ions, seems to be stronger, when possesing a catechol structure, that is two hydroxyl groups close to each other in either the A or the B ring of the flavonoid structure.

The structural components of the flavonoids which are proposed from many studies to be responsible for their antioxidative ability are: i) The number of hydroxyl groups in the rings, which can donate one electron ii) The C_4 keto group and iii) The C_2-C_3 double bond, which is necessary for spreading the negative charge through resonance effects (Rice Evans et al., 1996). Flavonoids themselves become free radicals in the process, but their conjugated structure allows the remaining orbital electron to be relatively inactive. The most powerful flavonoid antioxidant compounds according to TEAC value (Trolox Equivalent Antioxidant Activity) that have been reported are: i) Epicatechin gallate of the flavanol subfamily ii) Quercetin of the flavonol subfamily iii) The anthocyanidins delphinidin and cyanidin (Rice Evans et al., 1996). The flavones luteolin, apigenin,

chrysin and the flavonol kaempherol exhibit intermediate efficacies (Rice Evans et al., 1996).

1.2.3.3 Estrogenic and antiestrogenic activity

The estrogenic activity of isoflavonoids was first noted in the 1940s and since then a list of isoflavonoids have been shown to be agonists in various animal models (Birt et al., 2001). Miksicok in 1993 showed that commonly occuring flavonoids also possess estrogenic activity, by using an estrogen receptor dependent transcriptional response assay (Miksicek, 1993). The results published in this study revealed an estrogenic potential in the following order genistein> kaempferol> apigenin >luteolin > catechin> hersperitin (Miksicek, 1993). Compounds such as the above have been shown to protect against hormone related cancers. In a different study genistein showed growth inhibition of MCF7 cells at concentrations of 10 μM or greater, while at concentrations lower than 1 μM it stimulated growth (Le Bail et al., 1998). The authors proposed that this isoflavone can block the effects of oestradiol, even though it is itself oestrogenic.

The binding affinity to the oestrogen receptor of all the reported isofalvonoids or flavonoids seems to be weak, 103 to 105 fold less than 17β-oestradiol as reported from Davis et al in 1998 and depending on the concentration of natural estrogens they can be antiestrogenic if their circulating concentrations are 100-fold greater than estradiol (Birt et al., 2001). Oestradiol has a plasma concentration range of 200-1500 pmol/l in females (Gaw et al., 1999), hence it is possible for isoflavonoids to reach the concentration in which they would exert agonistic effects on the oestrogen receptor.

1.2.3.4 Antiinflammatory and related effects

Chronic inflammation can lead to genetic instability, via nitric oxide overproduction and subsequent generation of peroxynitrite which is a source of DNA damage. Thus flavonoids which act as antioxidants, have profound effects on the function of immune and inflammatory cells. Furthermore certain flavonoids can inhibit cell-cell interactions by possible effects on adhesion molecule function. Some may also interact directly with cytokines and cytokine receptors.

Some flavonoids can inhibit the enzyme NO synthase and therefore strengthen cellular protection against oxidative stress by blocking NO-associated DNA damage. The green tea polyphenol epigalocatechin gallate blocked the induction of NO synthase by down regulating NFκB activity in macrophages (Narayana et al., 2001). Moreover it was found that daidzein, an isoflavone, potentiates lymphocyte activation, suggesting that these effects may be involved in chemoprevention (Narayana et al., 2001).

1.2.3.5 Antiproliferation

It is well known that dietary flavonoids behave as general growth inhibitors. Although they appear non-toxic to human and animals they have been demonstrated to inhibit proliferationin many kinds of cultured human cancer cell lines. Kandaswamni and colleagues reported antiproliferative effects of flavonoids found in citrus fruits (quercetin, taxifolin, nobiletin and tangeretin) in the early 1990s on squamus cell carcinoma HTB43, whereas other studies have shown antiproliferative activities of flavonoids and isoflavonoids on A549 non small cell lung carcinoma and B16 melanoma cell lines (Kawaii et al., 1999). Strong inhibitory effects of flavone, flavanone and flavonol

subfamilies were also demonstrated in six different cancer cell lines with luteolin and 3',4'-dihydroxyflavone being the most potent (Fotsis et al., 1997). The IC_{50} values of the compounds examined in the above study were relatively high (> 40 μM) and only eriodictyol, luteolin, quercetin, and tangeretin had IC_{50s} of close to 10 μM or lower in the case of luteolin (Kawaii et al., 1999). Similarly Fotsis et al. found that chrysin, apigenin, luteolin and quercetin had IC_{50} values below 10 μM in SHEP human neuroblastoma and MCF7 cells.

1.2.3.6 Antimutagenic/Anticarcinogenic activity

Flavonoids may inhibit carcinogenesis by acting as "blocking agents" by one or more mechanisms. The most widely accepted mechanism is the inhibition of metabolic activation of a pro-carcinogen to its carcinogenic reactive intermediates. Several hydroxylated flavonoids, such as quercetin and myricetin, were found to inhibit the mutagenic activity of bay-region diol epoxides of B[α]P, which inhibited the tumorigenicity of –2-B[α]P-7,8-diol-9,10-epoxide-2 on mouse skin and in newborn mice (Middleton et al., 2000). Several flavones, flavonols and flavanones were shown to reduce the mutagenic activity of 2-amino-3-methylinidazo[4,5-f]quinoline (Middleton et al., 2000). The carbonyl at C-4, of the flavones was the main functional group required for this type of activity. Quercetin was further accessed by other groups for its ability to inhibit the initiation process of tumour promoters in the mouse skin carcinogenesis model and found to counteract the tumour promoter activity of TPA (Middleton et al., 2000). Quercetin also showed anticancer activity in various other animal studies. It inhibited hamster buccal pouch carcinogenesis and DMBA induced mammary cancer as well as azoxymethanol induced colon cancer in rats (Middleton et al., 2000). Finally various

naturally occuring flavonoids were shown to inhibit the activation of aflatoxin B1 to its AFB_1-8,9 epoxide (Middleton et al., 2000).

1.2.3.7 Antiadhesive/Antimetastatic properties

Fotsis and coworkers (1993) used a fractionation assay system by which they extracted flavonoids and isoflavonoids present in urine from healthy humans, who were fed a vegetarian diet. The extracts were then tested and it was found that genistein was a potent inhibitor of endothelial cell proliferation, stimulated by bFGF. Genistein also inhibited *in vivo* angiogenesis and TNF stimulated induction of endothelial cell adhesion molecules (Middleton et al., 2000). Other flavonoids investigated by Fotsis and coworkers were apigenin, luteolin, fisetin, 2′,3-dihydroxyflavone and 3′,4-dihydroxyflavone, which were shown to be antiangiogenic *in vitro*. The flavonoid (+)-catechin has been shown to bind laminin, which is an extracellular molecule involved in invasion and metastasis of tumour cells. The effect of laminin on the morphology and adhesion of two cell types MO4 and M5076 was abrogated by high concentrations of (+)-catechin (Middleton et al., 2000). Tangeretin and genistein inhibited the invasion of MO4 cells into embryonic chick heart fragments *in vitro* and of a BALB/c mammary carcinoma *in vivo* respectively (Middleton et al., 2000).

1.2.3.8 Effects on heat shock proteins

Heat shock proteins are induced usually due to heat stress and other stimuli and are involved in protein assembly and in other cell functions. Quercetin and other flavonoids inhibited the induction of heat shock proteins in HeLa cells (Middleton et al., 2000). In

the same cell line quercetin was found to be a hyperthermic sensitiser. This flavonoid was also shown to block heat shock protein synthesis in K562 cells at different levels depending on the temperature used and on the stressor employed. In human colon cancer cells treatment of quercetin in a concentration dependent manner abolished the development of thermotolerance, which was directly related to inhibition of heat shock protein synthesis.

1.2.3.9 Induction of detoxification enzymes

Another proposed mechanism for protection against cancer by flavonoids includes induction of Phase II metabolism enzymes. Many environmental carcinogens are oxidised to harmful metabolites and then detoxified by phase II enzymes into forms that are relatively inert and more easily excreted. There is significant evidence that cellular resistance to carcinogen exposure can be increased by induction of detoxifying enzymes. Two of the most important are quinone reductase and glutathione S transferase. Glutathione *S* transferase (GST) catalyses the conjugation of a variety of compounds with the tripeptide glutathione. Glutathione conjugation serves as a protective mechanism by which potentially toxic electrophillic metabolites are converted into less reactive compounds. Induction of GST has been noted for certain dietary antioxidant flavonoids, such as quercetin and epicatechin (Narayana et al., 2001). Moreover compounds such as B-naphthoflavone, which is a synthetic flavone derivative have been shown to induce GST (Narayana et al., 2001).

Similarly quinone reductase is a major enzyme in xenobiotic metabolism that catalyses two electron reductions and protects cells from potentially harmful carcinogenic species, such as free radicals, hydroperoxides and peroxide radicals generated by one electron reductions. It has been shown by Wang et al 1998 that this enzyme can be induced in a colonic Colo205 cell line, by isoflavones with genistein being the most potent inducer (Birt et al., 2001).

1.3 Cytochrome P450s

1.3.1 P450s in general

Cytochrome P450 is classified as a haem-containing enzyme with iron protoporphyrin IX as the prosthetic group. It is the terminal oxidase component of an electron transfer system, present in the endoplasmic reticulum responsible for the oxidation of almost all drugs. P450s contain haem b, which is not covalently bound to the polypeptide chain. The porphyrin ring provides four of the six ligands required for octahedral stereochemistry (four nirogen atoms). The fifth axial ligand is the thiolate group of a cysteine residue, compared to other haemoproteins, such as haemoglobin, where it is the nitrogen atom of a histidine residue, whereas the sixth axial co-ordination site is vacant. These enzymes are named after the characteristic γ (Soret) absorbance peak at 450nm, which is observed in the electronic spectra of their ferrous (Fe^{2+}) CO adducts and derives from cysteine axial ligation. In the cell P450s are located either in the smooth endoplasmic reticulum or in the mitochondria, since they require a membrane for their catalytic activity. They are composed of several domains; there is a hydrophobic *N*-

terminus, which acts as a membrane anchor for the P450s; a substrate binding site; an oxygen binding site; and a free hydrophillic C terminal.

Cytochrome P450 is not a single enzyme, but rather consists of a family of closely related enzymes and exists as multiple forms of monomeric molecular weight of the range 45,000-55,000 Daltons (Gibson and Skett, 2001). To date over 36 gene families have been identified, 12 of which exist in all mammals and this includes over 800 cytochrome P450 genes, encompassing all of the major phyla, such as animalia, plantae, fungi and monera kingdoms (Gibson and Skett, 2001). This resulted in the construction of a commonly agreed nomenclature system, in order to avoid confusion regarding the identity of individual enzymes. For example the nomenclature of CYP2C9 is as follows: The CYP abbreviation refers to the protein which is encoded by the *cyp* gene. The first numeral refers to the family with members of the same family having more than 40% amino acid sequence homology. The capital letter refers to the subfamily with members of the same subfamily having more than 55% aminoacid sequence homology. The last numeral refers to the isoform. Table 1.7 gives an account of the mammalian cytochrome P450 families.

Table 1.7 Mammalian Cytochrome P450 families. Adapted from Gibson and Skett 2001

P450 family	No. of subfamilies	No. of isoforms	Reactions
CYP1	2	3	Drug metabolism
CYP2	8	58	Drug and steroid met.
CYP3	2	10	Drug and steroid met.
CYP4	6	20	Fatty acid ω hydroxylations
CYP5	1	1	Thromboxane synthase
CYP7	1	1	Cholesterol $_{7\alpha}$ hydoxylase
CYP11	2	4	Steroid $_{11\beta}$ hydoxylase
CYP17	1	1	Steroid $_{17\beta}$ hydroxylase
CYP19	1	1	Aromatase
CYP21	1	2	Steroid $_{21}$ hydroxylase
CYP24	1	1	Vitamin $D_{3\ 25}$ hydroxylase
CYP27	1	1	Cholesterol $_{27}$ hydroxylase

Currently there are about 60 human P450s and analysis of the human genome sequence has indicated that it is unlikely to be any more. Functionally there are two broad classes of P450, those who metabolise xenobiotics (foreign compounds) and includes CYP1, CYP2, CYP3 and to a lesser extent CYP4 and those who metabolise endogenous compounds such as cholesterol and steroid hormones and includes CYP5, CYP7, CYP11, CYP17, CYP19, CYP21, CYP24 and CYP27. The xenobiotic metabolising forms of

P450 are also capable of metabolising endogenous compounds and thus possess other endogenous functions.

The liver is the main normal tissue in which P450s are expressed. CYP1A2, CYP2A6, CYP2C, CYP2D6, CYP2E1 and CYP3A4 are all present in the liver. CYP3A4 is the predominant CYP enzyme and accounts for about 30% of the total human hepatic P450. However individual cytochrome P450 forms are also expressed in specific extrahepatic tissues, including small intestine kidney and lung.

P450s catalyse a variety of chemical reactions. The most important and intensively studied drug metabolism reaction is the Mixed Function oxidase (MFO) reaction. The general equation for the reaction is as follows:

$$NADPHH^{+} + O_2 + RH \rightarrow NADP^{+} + H_2O + ROH,$$

where RH can be an oxidisable drug substrate and ROH the hydroxylated metabolite. During this reaction one atom of oxygen is incorporated in the metabolite and the other atom is reduced to water. The MFO system also requires another enzyme called NADPH cytochrome P450 reductase, a flavoprotein consisting of flavin mononucleotide (FMN) and one flavin adenine dinucleotide (FAD). The central features of the cytochrome P450 catalytic cycle are outlined in Figure 1.21 and can be summarised in six steps.

Step 1: This step involves binding of the substrate to the ferric state (Fe^{3+}) of P450. In the free (resting) enzyme the sixth co-ordination site is occupied by water and this results in a stable low spin configuration with a reduction potential of –300mV. Upon binding of the substrate near the heme, the water ligand is lost from the Fe^{3+}, which then becomes high spin with an increase in reduction potential to –170mV. The substrate does not ligate

the Fe^{3+} but it is held in a binding pocket interacting by various means, such as hydrophobic interactions and hydrogen bonds.

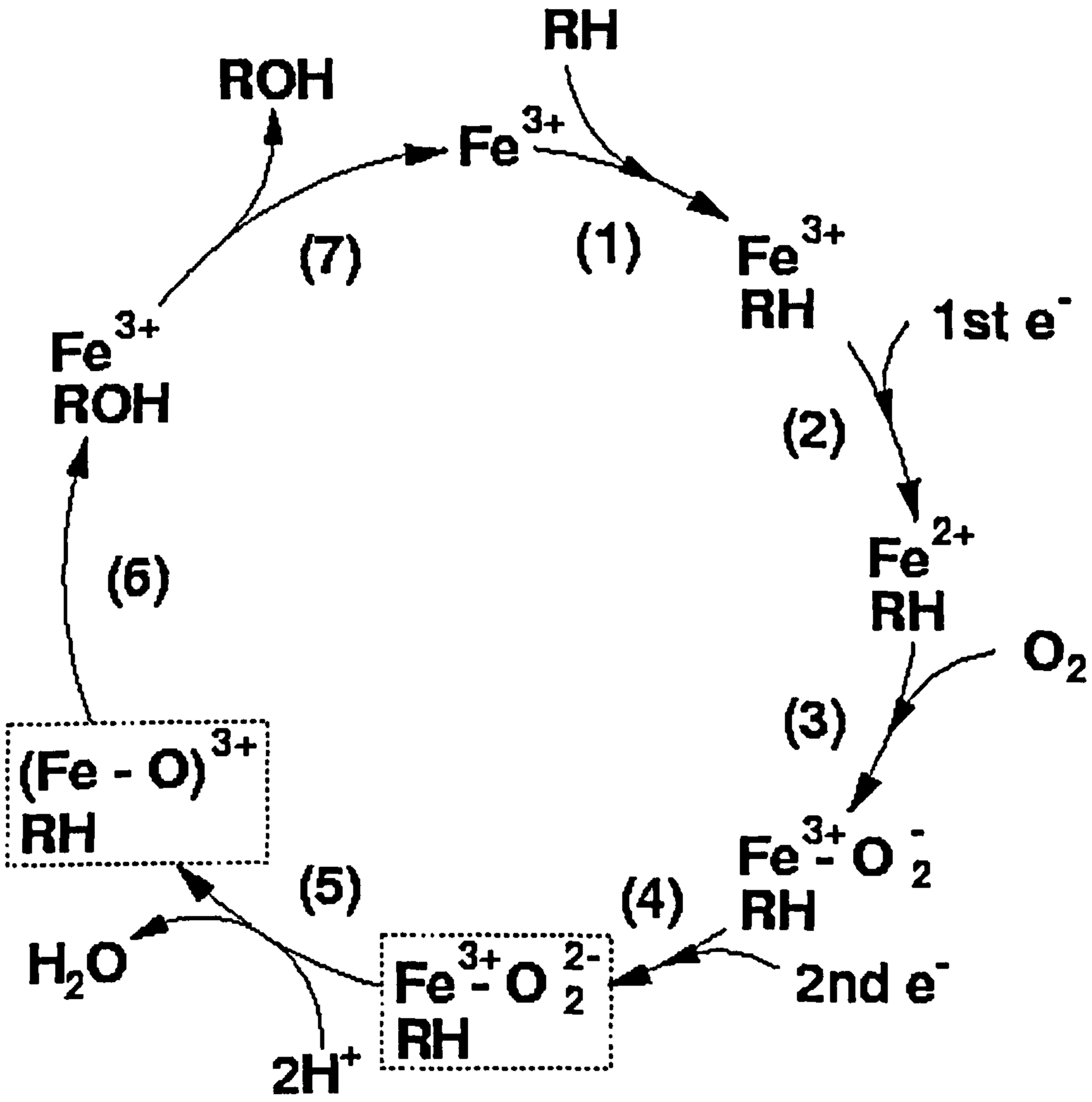


Figure 1.21 The catalytic cycle of cytochrome P450

Step 2: This involves the first electron reduction of substrate-bound ferric cytochrome P450 to the ferrous state (Fe^{2+}). Raising the reduction potential upon substrate binding makes the Fe^{3+} more easily reducible to Fe^{2+}. The electron required for this reduction is initially derived from NADPHH+ and is transferred by the flavoprotein NADPH-cytochrome P450 reductase.

Step 3: This step involves binding of molecular oxygen to the ferrous substrate P450 complex. This reaction is not well characterised in the mammalian system, due to unstable nature of oxyferrous substrate complex.

Steps 4,5 and 6. These steps involve electron rearrangement, introduction of the second electron and subsequent oxygen insertion in the substrate with hydroxylated metabolite release. Step 5 involves the input of the second electron, usually derived from NADPH-cytochrome P450 reductase. Step 6 involves two steps namely abstraction of a hydrogen atom from the substrate and oxygen rebound. The oxidation states of iron in the intremediate states are not precisely known, but are thought to involve metal to ligand charge transfer, such that the complex is low spin ferric iron with superoxide. After the second electron is passed, the newly formed $Fe^{2+}/O_2^{-\bullet}$ intermediate can undergo further metal to ligand charge transfer to give ferric peroxide substrate bound P450 Fe^{3+}/O_2^{-2}, which rapidly decays to give water and a Fe^{5+} oxo compound, a seven co-ordinate Fe^{5+} complex, with O^{2-} acting as a bidentate ligand. This preferentially exists as the sixth co-ordinate radical species Fe^{4+}-$O^{-\bullet}$, which reacts with the substrate to give hydroxyferryl intermediate and substrate radical. Finally via the rebound mechanism the hydroxyferryl species reacts with the substrate radical to give hydroxylated substrate and five co-ordinate ferric heme.

These enzymes are very powerful catalysts, since in the absence of P450 this oxidation requires a very high temperature. The usual outcome of P450 metabolism is activation or inactivation of the substrate; paradoxically sometimes P450 can lead to either acute or chronic cellular toxicity (Patterson and Murray, 2002).

1.3.2 CYP1 family enzymes

The human *CYP1* gene family, which is one of the major cytochrome P450 involved in the metabolism of xenobiotics is now known to consist of three individual forms classified into two subfamilies. The CYP1A subfamily contains two well characterised but distinct members, CYP1A1 and CYP1A2. CYP1A1 is primarily expressed in extrahepatic tissues, whereas CYP1A2 is a major P450 expressed in the liver, accounting for approximately 15% of total P450 activity. A second human CYP1 subfamily was identified almost 10 years ago, which to date contains only one member CYP1B1. Sequence analysis shows about 40% homology of CYP1B1 with both CYP1A1 and CYP1A2 (Kress and Greenlee, 1997).

Murray et al. in 1997 showed with immunohistochemical studies, that CYP1B1 is expressed in a wide variety of tumours and only in 5 out of 127 tumor samples CYP1B1 was not detected. In every type of cancer CYP1B1 immunoreactivity was localised specifically to tumour cells with non tumour cells, including inflammatory and endothelial cells present in the sections of the tumour, showing no immunoreactivity (Murray, 2000). The samples tested were primary malignant tumours of bladder, brain, breast, colon, connective tissue, oesophagus, kidney, liver, lung, lymph node, small intestine, testis, stomach and uterus tissues (Murray et al., 1997). Elevated expression of CYP1B1 has also been reported in primary ovarian cancer, (92% of 167 samples tested) with the enzyme being localised in the cytoplasm (McFadyen et al., 1999). The same study showed overxpression of CYP1B1 in metastatic ovarian cancer (94% out of 48 samples). The overexpression of CYP1B1 in colon, lung and breast cancer has been confirmed by various other studies (Murray, 2000, McFadyen et al., 2001). It has also

been shown that CYP1A1 is expressed in breast cancer but to a lesser extent (25%) (McKay et al., 1995). However other studies show that CYP1A1 mRNA is found to a larger extent in breast tumours (possibly because of the smaller number of samples being tested) and also that CYP1A1 mRNA is present in normal breast tissues (Huang et al., 1996). CYP1A1 activity was identified in colon cancer and in normal colon, whereas in another study CYP1A overexpression was identified in colon cancer with no detection of such expression in normal tissues respectively. CYP1A1 mRNA has been identified by northern hybridisation in lung cancer (Patterson and Murray, 2002) and CYP1A1 activity has also been detected in normal small intestine in small amounts. CYP1A1 and CYP1A2 have been shown to be expressed in squamus cell carcinomas (Nakajima et al., 1991). The same enzymes were detected in both hepatocellular carcinoma and in normal liver by another study, but the authors suggested a possible downregulation of CYP1A expression (McKinnon et al., 1991).

1.3.3 Carcinogen activation hypothesis

Since the CYP1 family enzymes and mostly CYP1A1 and CYP1B1 have been shown to be expressed in a variety of tumors and bearing in mind that they are involved in the metabolism of xenobiotic substances, a lot of studies have related them to carcinogenesis. It has been proposed by certain studies that CYP1 family enzymes are capable of activating procarcinogens and mutagens by forming reactive intermediates from compounds such as polycyclic aromatic hydrocarbons (PAH) (Shimada et al., 1996, Kim et al., Kress, 1997). The most commonly encountered members of PAH family in the literature include benzo[α]pyrene, 3-methylchloranthrene and 7,12-

dimethylbenzanthracene. *In vitro* metabolism of benzo[α]pyrene by CYP1 enzymes leads to the formation of the ultimate carcinogens diol epoxide, phenol metabolites and quinone metabolites (Kim et al., 1999). Phenol metabolites are less mutagenic compared to quinone, since they can be detoxified by Phase II enzymes. Diol epoxides have been shown to cause mutations by attacking the p53 tumour suppressor gene in normal bronchial epithelial cells (Kim et al., 1999). The same epoxides have been shown to induce mutations in proto-oncogenes involving cell proliferation such as H-ras. CYP1 family enzymes have also been shown to catalyse the metabolism of aryl amines to carcinogenic compounds, with CYP1A2 being highly active in catalysing several aryl amines, particularly 2-acetylaminofluorene, 2-amino-3-methylimidazo[4,5-f]quinoline, 2-amino-6-methyldipyridol and 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline and CYP1B1 being the most active in catalysing activation of 2-aminoanthracene (Shimada et al., 1996).

PAHs are not only substrates for CYP1 family enzymes but they have also been shown to induce the expression of these enzymes through a specific cytosolic receptor termed the aryl hydrocarbon receptor (AhR). As shown in Figure 1.22 PAHs enter the cell and combine with cytosolic AhR. AhR in its free form exists with other proteins Hsp90 and AhR interacting protein (AIP). Upon binding with the inducer these two proteins dissociate with AhR and AhR-induced complex is translocated to the nucleus. There it binds with another protein called AhR nuclear translocator protein (ARNT) and forms a heterodimer. The inducer-AhR-ARNT complex targets a number of genes in the nucleus. This complex interacts with specific gene regulatory sequences called xenobiotic responsive elements (XREs) of the sequence 5′-TNGCGTC, which function as

transcriptional enhancers and negative control elements, situated at approximately 1 kilobase of the transcriptional start site in the 5′ flanking region of the gene. This initiates transcription of *CYP1* family genes (Gibson and Skett, 2001).

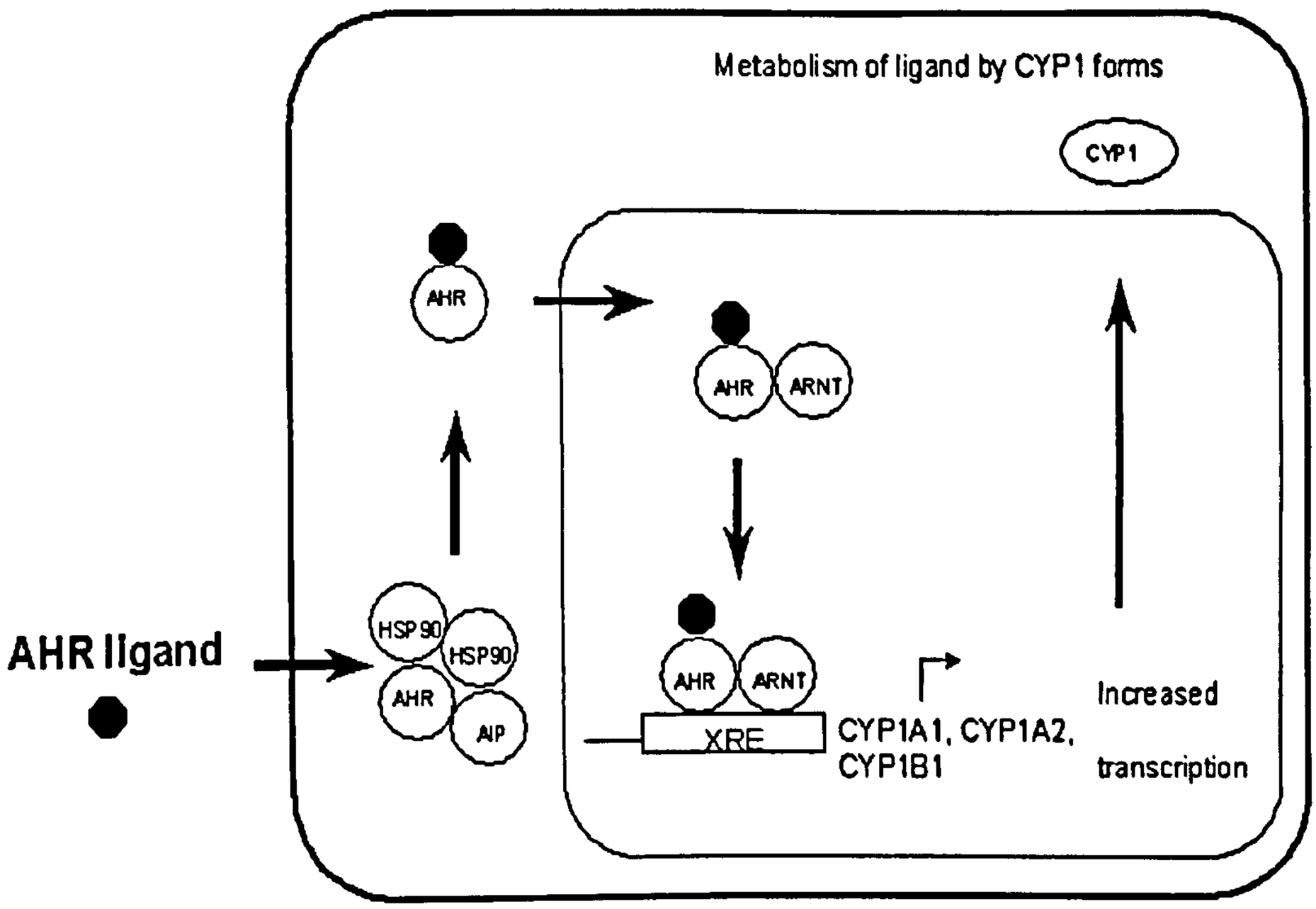


Figure 1.22 Receptor mediated induction of CYP1 family enzymes

The AhR is a cytosolic protein of molecular weight of 88kDa and is a member of the family of the basic helix-loop-helix transcription factors. It consists of several domains, such as ligand binding and DNA binding. It is important to note that the AhR/ARNT complex is involved in the induction of other genes besides the *CYP1* family, which

encode for other xenobiotic metabolising enzymes, such as GST and UDP-glucuronyl transferase enzymes (Gibson and Skett, 2001). The ARNT is a nuclear protein of molecular weight of 86 kDa and again consits of distinct functional domains responsible for DNA binding, dimerisation and nuclear localisation (Gibson and Skett, 2001).

The compound that has been extensively used for the induction of CYP1 family enzymes from various studies is 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD). TCDD elicits a diverse spectrum of biological and toxicological responses. It induces CYP1A1 expression in HepG2 cells via AhR mediated mechanism (McKinnon et al., 1991). CYP1B1 was constituvely expressed in human renal adenocarcinoma cell line (ACHN) and the expression was increased after induction with TCDD 8-fold, whereas no induction was seen in HepG2 cells. In early passage human-mammary epithelial cells (HMECs) isolated from reduction in mammoplasty tissue of seven individual donors very high levels of AhR and ARNT protein were reported to be expressed (Larsen et al., 1998). CYP1B1 was shown to be constituvely expressed in HMECs as well, whereas CYP1A1 expression could be induced by TCDD (Larsen et al., 1998). A comparison of protein expression levels of AhR and ARNT between the breast cancer cell line and human-mammary epithelieal cells revealed that the level of AhR expression was many time higher in cultured HMECs as compared to MDA-MB 231 cells (Larsen et al., 1998). The same study showed that CYP1B1 and CYP1A1 demonstrated significant levels of 7,12- dimethylbenzanthracene (DMBA) metabolism, in enzyme assays, with CYP1A1 being 6-fold more efficient. TCDD induces the expression of CYP1 enzymes in a variety of other cell lines (Larsen et al., 1998, Jana et al. 1999, Dohr et al., 1995).

CYP1 family isozymes are further involved in the metabolism of oestrogens, besides PAHs and related compounds. It is known that the oestrogens 17β-oestradiol (E2) and oestrone (E1) are metabolised via two major pathways: formation of the 2- and 4-catechol oestrogens and 16α hydroxylation. CYP1B1 catalyses the C-4 hydroxylation of oestradiol to the corresponding catechol, a product shown to be carcinogenic in animal models (Kristensen and Borresen-Dale, 2000). Human breast tissue expresses high levels of CYP1B1 and as a consequence breast cancer has higher levels of 4-OH oestradiol than normal breast tissue. CYP1A1 also catalyses the C-2, C-4, C-6α and C-15α hydroxylation of oestradiol favouring the C-2 hydroxylation compared to C-4 for CYP1B1 (Badawi et al., 2001). CYP1A2 catalyses C-2, C-4, C-16α hydroxylation and favours C-16α and C-2 hydroxylation of oestradiol (Badawi et al., 2001). Unless detoxified catechol oestrogens may be oxidised to electrophillic metabolites, catechol oestrogen quinones that can react with DNA to form depurinating and stable adducts. These adducts, particularly depurinating adducts, can lead to oncogenic mutations and may subsequently initiate many human cancers. Thus it can be hypothesised that induction and subsequent expression of CYP1 family enzymes may lead to increased synthesis of catechol oestrogens and depurinating DNA adducts. There are several studies which have demonstrated increased rates of formation of C-2, C-4 and C-16α hydroxylated metabolites of E2 after induction of CYP1 family enzymes by TCDD and related compounds via AhR either in MCF7 cells or in animal models and suggest that these may be factors contributing to the biological effects associated with exposure to these agents (Badawi et al. 2000, Badawi et al. 2001, Morgan et al., 1998, Spink, 1998). Other studies have related the catalytic activity of these enzymes to endocrine regulation

and increased toxicity of oestrogens which can eventually lead to the formation of oestrogen dependent tumors (Hayes et al., 1996, Liehr and Ricci, 1996). It has also been reported that the oestrogen C-4 hydroxylase activity in breast cancer cells can be used as a potential tumor marker in combination with C-2 hydroxylase activity (Liehr and Ricci, 1996). An elevated ratio of 4/2 hydroxy oestradiol formation in neoplastic mammary tissue may be a useful marker of benign or malignant breast tumors (Liehr and Ricci, 1996).

1.3.4 Rescue enzyme hypothesis

P450s are believed to have existed since the beginning of life over 3.5 billion years ago, but the P450s responsible for foreign compound metabolism appear to have arisen about 400 to 500 million years ago and it is believed that these enzymes were needed to metabolise and detoxify chemicals found in plants(reviewed in Gonzalez and Gelboin, 1994). In the plant-animal "warfare" hypothesis plants produce toxins to kill predators and animals evolve P450s to detoxify these toxins and as this process continues over millions of years new catalytic activities of P450s will develop (reviewed in Gonzalez and Gelboin, 1994).

Similarly, one can speculate that in the field of chemical carcinogenesis the P450s and other foreign compound metabolising enzymes would evolve for a purpose beneficial to the animal. Indeed, the gene sequence of CYP1B1 has been determined and it is thought that the CYP1B branch may have separated from CYP1A about 300 million years ago (reviewed in Gonzalez and Gelboin, 1994).

Although CYP1B1 has been implicated in carcinogenesis, this does not adequately explain the cause of tumours, since there are many mutagenic origins and oncogenic transformations that result in various types of cancer, and yet CYP1B1 appears to be present in a wide variety of tumours irrespective of their oncogenic origin (Murray et al., 1997). CYP1B1 has been proposed to be a tumour suppresor enzyme, since it was shown to catalyse the formation of picetannol, a stilbene which possesses antileukaemic activity, from resveratrol, a cancer preventative agent found in red wine (Potter et al., 2002). The authors of this study suggest that with respect to carcinogenesis, it does not matter if carcinogens are activated in cancer cells, since they are already cancerous. Relevant observation to support such a hypothesis is the fact that CYP1B1 can be induced in skin cells on exposure to mutagenic UV light. CYP1B1 and the CYP1A subfamily enzymes can be induced via the AhR in an alternative way as to that previously described (PAHs and related compounds). The genes encoding for CYP1A and CYP1B1 proteins, are controlled by an inducible transcriptional complex termed the hypoxia inducible factor-1 (HIF-1) (Blancher and Harris, 1998, Wood et al., 1996). HIF-1 is a heterodimeric DNA binding complex consisting of two basic helix-loop-helix (bHLH) PAS (Per-ARNT-Sim) proteins HIF-1α and HIF-1β (Blancher and Harris 1998, Wood et al., 1996). HIF-1β is actually the ARNT the dimerisation protein of AhR. HIF-1α is only induced under hypoxic conditions, where it dimerises with ARNT and the resulting complex induces the transcription of CYP1 family genes by binding to the relevant responsive elements (HREs or XREs) (Blancher and Harris, 1998, Wood et al., 1996). One of the major driving forces of tumour progression is hypoxia. The vasculature of tumours is relatively poor, which leads to decreased blood perfusion and uptake of oxygen by the cancer cells.

These low oxygen conditions may play an important role in cellular metabolism and may be the stimulus for induction of CYP1B1 and the other CYP1A enzymes as a defense mechanism against tumor promotion.

1.4 Aims

The purpose of the work described in this thesis was to examine whether, dietary flavonoids can prevent cancer through inhibition of CYP1 family enzymes (Chapter 3), thus preventing activation of pro-carcinogens, or through selective CYP1 enzyme mediated metabolism (Chapters 4,5,6), leading to formation of more cytotoxic species than the parent compound. The ability of dietary flavonoids to induce CYP1 family enzyme expression (Chapter 7) and therefore stimulate their own metabolism in cancer cell lines was also investigated. In addition with the above aims this would lead to a more conclusive concept of how dietary flavonoids interact with CYP1 family enzymes and might provide evidence for a new mechanism of their chemopreventative action.

2.MATERIALS AND METHODS

2.1 Chemicals and enzymes

Apigenin (95% pure), luteolin (98% pure), quercetin (98% pure), myricetin (85% pure), kaempferol (90% pure), chrysin (96% pure) and baicalein (98% pure) were from Sigma (Poole UK), diosmetin, scutellarein and sinensetin (99% pure) from Indofine (Hillsborough NJ, USA) and eupatorin, eupatorin-5-methyl ether, cirsiliol and genkwanin (97% pure) from Lancaster (Heysham UK). Stock solutions were prepared in dimethyl sulfoxide and stored at -20°C. 7-ethoxy resorufin was purchased from Sigma (Poole UK), prepared as 1 mM stock solution in dimethylsulfoxide and kept at 4°C. CYP1 supersomes were purchased from BD Biosciences (Cowley UK). They are microsomes derived from baculovirus infected cells (www.bdbiosciences.com). Solvents for the HPLC analysis were of HPLC grade and purchased from Fisher (Loughborough, UK). Phosphate buffer reagents, $NADP^+$, Glucose-6-Phosphate, Magnesium Chloride, Glucose-6-phosphate dehydrogenase, NADPH, FCS, EGF, Insulin, Hydrocortisone, DMEM, MTT, RPMI, PI, Rnase, DTT, western blotting RNA extraction, RNA and DNA gel electrophoresis reagents were obtained from Sigma (Poole UK). ECL was purchased from Amersham (Little Chalafont UK). All reagents except the HPLC solvents, tissue culture and molecular biology reagents were of analytical grade.

2.2 Cell lines

MDA-MB 231 was derived from pleural effusion of a Caucasian 51 year old female with adenocarcinoma of the breast (www.lgcpromochem-atcc.com). MDA-MB 468 was derived from pleural effusion of a Black 51 year old female with metastatic adenocarcinoma of the breast (www.lgcpromochem-atcc.com). MCF7 was derived from

a Caucasian 69 year old female with breast adenocarcinoma, whereas MCF10A was derived from the mammary gland of a 36 year old Caucasian female with fibrocystic disease (www.lgcpromochem-atcc.com). MCF10A cells were grown in serum-free medium with low Ca^{++} concentrations and lost their ability to undergo senescence (www.lgcpromochem-atcc.com).

2.3 EROD and phosphate buffer solutions

Solution A was prepared by mixing $NADP^{+}$ (20 mg/ml), Glucose-6-Phosphate (20 mg/ml) and Magnesium Chloride (13.3 mg/ml). Typically a 10 ml solution was prepared, which was aliquoted to 0.5 ml solutions that were stored at –20°C. Solution B was prepared by diluting Glucose-6-phosphate dehydrogenase (G6PDH) with phosphate buffer to a final concentration of 40 U/ml. The solution was aliquoted to 0.1 ml solutions, which were stored at –20°C. Solution C was prepared freshly by mixing solutions B and A with phosphate buffer (100 mM) and water to a ratio of 1:5:5:39 respectively. 7-ethoxyresorufin solution was prepared as a stock solution by diluting 1 mg of compound with 4.15 ml of DMSO to a final concentration of 1 mM. The solution was kept at 0°C. Phosphate buffer for EROD assay and drug metabolism experiments was prepared by diluting 8.375 g disodium hydrogen orthophosphate and 5.579 g of potasium dihydrogen orthophosphate in 1 L of water. The pH of the solution was adjusted to 7.4 with additions of small portions of NaOH (1M). The solution was finally stored at 4°C.

2.4 7-Ethoxy resorufin-*O*-deethylase activity

7-Ethoxy resorufin-*O*-deethylase activity of the three CYP1 enzymes was determined using continuos fluorimetry with NADPH as a cofactor (Burke et al., 1994).

Inhibition of ethoxy resorufin-O-deethylase activity. The reactions were carried out in 96-well plates and each incubation in each well (100 µl) contained supersomes (5 pmol/ml), solution C ($NADP^+$ 1.3 mM, Glucose-6-phosphate 3.3 mM, $MgCl_2$ 3.3 mM and glucose-6-phosphate dehydrogenase 4 U/ml), 7-ethoxyresorufin (5 µM), phosphate buffer (30 mM) and inhibitor as appropriate. For an initial screen five concentrations of inhibitor were achieved (25, 2.5, 0.25, 0.025, 0.0025 and 0 µM). A background was also prepared containing phosphate buffer, supersomes and solution C. Reactions were initiated with the addition of supersomes, carried out for 20 min at 37°C and were terminated by the addition of 100 µl of methanol. The samples were cooled in ice and fluorescence was measured at excitation λ 530 nm and emission 590 nm. The results were plotted as percentage of inhibition compared to control against inhibitor concentration, using Prism software. IC_{50} were calculated from the graph obtained from each inhibitor.

2.5 Flavonoid metabolism

Incubations (100 µl) contained supersomes (20 pmol/ml CYP1B1, CYP1A1, CYP1A2) NADPH (0.5 mM) $MgCl_2$ (0.5 mM), phosphate buffer (20 mM) and flavonoid (10 µM). Following incubation at 37°C, samples were taken at 5 minute intervals until 20 minutes. On sampling reaction was terminated by the addition of 100 µl of methanol:acetic acid mixture (100:1). Incubates were centrifuged at 3,500 g for 20 minutes at 4°C. Supernatants were analysed by reverse phase HPLC according to the following method:

A Luna C18 4.6 x 150 mm 5 μ column was used. Solvent A contained water, 1% acetonitrile and 0.5% acetic acid and solvent B contained methanol, 4% acetonitrile and 0.5% acetic acid. The following gradient program was used: 60% solvent A and 40% solvent B at time 0, 10% solvent A and 90% solvent B at time =10 minutes. Final conditions were held for 1 minute until returning to initial solvent composition conditions. 8 minutes were left for column reequilibration after each run. Detection was monitored by UV between 325-350 nm depending on the compound used and the assay was carried out at 37°C. Flow rate was 1 ml/min.

2.6 Kinetic experiment

The same methodology as **2.4** was followed. The incubation time and enzyme concentration were standardised by preliminary experiments (usually 5-10 min and 5-10 pmol/ml of CYP), depending on each compound and a concentration range of flavonoid was achieved in each reaction (10, 8, 4, 2, 1, 0.5, 0.25 μM).

2.7 Cell culture and the maintance of cell lines

MCF-7 and MDA-MB 468 cells were maintained in RPMI 1640 with and without phenol red respectively. The use of phenol red-free medium is advised when steroid hormone effects are being studied, because phenol red can act as a oestrogen receptor agonist (Davis JM, 2002). MDA-MB 468 cells have been shown to be oestrogen receptor negative (Spink DC et al., 1998), so using a phenol red-free medium would ensure that they would retain this pattern of gene expression. The medium also contained Glutamax 1 (Life Technologies) and 10% (v/v) heat inactivated foetal calf serum. The cells were

grown at 37 °C, 5% CO_2 /95% air with 100 % humidity and passaged using trypsin EDTA. MCF10A cells were maintained in DMEM at 37 °C, containing Glutamax 1, 10% (v/v) heat inactivated foetal calf serum as above , EGF (20 ng/ml), Insulin (10 µg/ml) and hydrocortisone (500 ng/ml). MDA-MB 231 cells were maintained in the same conditions as MCF7 cells.

2.8 MTT cytoxicity assays

2×10^3 MCF7 cells were plated in 100 µl of medium in 96-well flat-bottomed plates. After 4 hrs to allow maximum cell adherance, 100 µl of medium containing TCDD, or medium with 0.2% (v/v) DMSO as control were added to the wells to give a final concentration of 10 nM or 0.1% (v/v) DMSO. After 24 hrs the medium was carefully aspirated and 100 µl of fresh medium was added. Within 30 minutes compound was added in quadruplicate in 100 µl medium to give a final concentration of not more than 0.1% (v/v) DMSO. The cells were then allowed to grow for 4 days to give 80-90% confluence in the control wells. 50 µl MTT (2 mg/ml in PBS) was then added to each well for 3 hrs. All medium was aspirated and the formazan product generated by viable cells was solubilised with 150 µl of DMSO. Plates were vortexed and the absorbance at 540 nm determined using a UV plate reader. Results were expressed as the percentage of 100% (control) proliferation and the IC_{50} calculated using Graphpad Prism software. Dose range generally was in half log dilutions e.g. 100, 30,10, 3, 1, 0.3, 0.1, 0.03, 0.01, 0.003, 0.001 µM. In the case of MDA-MB 468 and MCF10A cells the assay was performed as above with the exception that no TCDD was added to induce the cells. For

inhibition studies, inhibitor was added to a final concentration of 2 μM in 200 μl of medium along with the compound.

2.9 Metabolism in cell lines

2×10^4 cells/ml were plated in 24-well flat bottomed plates and left to grow for 48 hours. MCF7 cells were preinduced with 10 nM of TCDD 24 hr prior to the experiment. The medium was aspirated and the cells were washed once with PBS. Compound was added in 200 μl of medium at a final concentration of 10 μM. At least three wells of the plate, from each cell line were kept with no compound for protein determination via the Bradford assay (Bradford, 1976). The protein concentration of the cells, was estimated approximately to be 0.5 mg/ml. The cells were incubated at 37°C for 45 minutes. The medium was then removed and the compound was extracted with the addition of 1% acetic acid in methanol. 200 μl of reporter lysis buffer was added to each of the wells and the cells were left for 30 minutes at –20°C. The cells were then thawed and with the aid of a scraper detached from the bottom of the wells. 200 μl of 1% acetic acid in methanol was added to the cell lysates. Both cell and medium samples were centrifuged for 15 minutes at 13,000 rpm and finally analysed with HPLC.

2.10 Western blotting

MDA-MB 468 and MCF10A cells were trypsinised and resuspended in 200 μl lysis solution (Sigma, Poole UK) containing protease inhibitor cocktail (Sigma, Poole UK) and DTT (1 mM). The protein concentration of that sample was estimated by the Bradford

assay (Bradford MM, 1976), so that it would not exceed 0.8 mg/ml per sample. 20 µl of that sample were mixed with SDS PAGE Laemmli buffer (Laemmli UK, 1970) (containing 5% mercaptoethanol) in 1:1 ratio. The sample was heated at 100 °C for 4-5 minutes and then 40 µl were loaded onto the gel. Two types of gel were prepared for each gel holder. First the running gel (10%) was placed followed by 0.5 ml of 50%, which would create a distinct line, between the running and the stacking gel. After 20 minutes the gel was set, the butanol removed and the stacking gel was added on top along with the casting comb. The running and stacking gel were composed of 7.9 and 2.5 ml H_2O, 6.7 and 0.625 ml of 30% acrylamide, 5 and 1.05 ml of 1.5 M Tris·Cl (pH 8.8), 0.2 and 0.04 ml of 10% Sodium Dodecyl Sulfate (SDS) , 0.2 and 0.02 ml of 10% Ammonium persulfate (APS), 20 and 4.5 µl of TEMED (*N*-,*N*- tetramethylethylenediamine) respectively. After some time the combs were removed so that the samples could be loaded on the gel. Inside and outside chambers were filled with 1 X running buffer to the top and 1/3 of the total volume respectively. The running buffer was prepared as 5 X stock by mixing 75.5 g of Tris, 360 g of Glycine and 25 g of SDS with 5 L of H_2O. The gel was left to run for 10 minutes at 150 V and for 1 hr at 110 V. The gel was then removed from the glass. The stacking gel was separated from the running gel and thrown away. The running gel was soaked in Cathode Buffer. Three pieces of blotting paper, which were two times larger than the size of the gel, were soaked in Cathode buffer. They were placed one on top of the other on the semi-dry blotter and rolled out to remove any air bubbles. The Gel was placed on top of the blotting paper. Nitrocellulose membrane was soaked in Anode buffer II and placed on top of the gel. Three pieces of blotting paper, which had the same the size of the gel, were soaked in Anode buffer II and placed

on top of the membrane. Another three pieces of the same size blotting paper were soaked in Anode buffer I and placed on top of the previous paper. All the bubbles were removed carefully by rolling out the papers with a disposable pipette tip. Anode buffer I, II and Cathode buffer contained 300, 25 and 25 mM Tris, and 20% methanol each. The pH of Anode buffer I and II was adjusted to 10.4 and Cathode buffer also contained 40 mM of 6-aminohexanoic acid. The semi dry blotter was left to run at a constant current of 0.8 mA/cm^2 of gel surface to nearest 10 mA for 1.5 hr. The membrane was removed and washed in PBS/0.1% Tween for 10 min with gentle shaking. The membrane was marked, so as to determine the protein side. The PBS/0.1% Tween was poured off and 10% milk was added. The volume was enough just to cover the surface of the membrane. The membrane was left at room temperature for 1 and ½ hr with gentle shaking. The membrane was then incubated overnight at 4 °C in 1% milk in PBS/0.1% Tween, containing primary antibody (1:1,000 dilution, Gentest IgG raised in rabbit, lot number 3) with gentle shaking. The next morning the membrane was rinsed briefly with PBS/0.2% Tween and washed three times with PBS/0.2% Tween for 10 minutes each. Secondary antibody (Sigma, antirabbit IgG) was diluted in 1% milk containing PBS/0.1 % Tween and added to the membrane for 1 hr at room temperature with gentle shaking. The secondary antibody solution was then poured off and the membrane was rinsed briefly with PBS/0.2% Tween. The membrane was then washed 2 times for 10 minutes with PBS/0.2% Tween, followed by washing two times for 10 minutes each in PBS with gentle shaking. The membrane was finally rinsed twice with sterile H_2O. The membrane was then placed on cling film and 1.5-2 ml of ECL detection reagents were added on it dropwise. The membrane was left for 1 min and then the excess liquid was removed. The

membrane was then placed on a new piece of cling film and inserted into the cassette. A piece of Kodak film was exposed for 10-30 minutes. The film was then placed in the following solutions for specific time points: Developer (4 min), stop bath (1 min), fixer (3 min) and running water (5 min).

2.11 FACS instrumentation

The flow cytometer is a machine that accepts an input of liquid suspended cells, passes them individually through a light source and measures a variety of light scattering and fluorescent properties for each cell. Input cells are passed through a capillary at a rate of thousands per second and subjected to light one by one in the form of a laser beam (Hughes and Mehmet, 2003).

To analyse each cell of a sample suspension through the laser beam, the cells must be first separated from each other and the laser beam must be small enough, so that two cells cannot be in the beam at the same time. The laser beam is focused to a very narrow cross section and the stream is also focused using "hydrodynamic focusing" (Hughes and Mehmet, 2003). Sheath fluid, which is usually PBS flows through a constricting nozzle, called the flow cell. The suspension of the sample is injected into the middle of the sheath through a small tube called the sample insertion rod. The flow of the suspension and the sheath fluid is laminar and the fluids do not mix. The sample stream (sample core) is surrounded by the sheath stream as the fluids pass through the flow cell.

Once the sample core comes out of the flow cell it is subjected to a laser beam and the resulting fluorescent and light scattering properties are measured by a number of sensors. The first two are postioned at 180° and 90° from the light source and called forward and

side scatter respectively (Hughes and Mehmet, 2003). Forward scatter reflects the cell size and side scatter reflects cellular morphology, granularity and membrane complexity (Hughes and Mehmet, 2003). These measurements provide information about morphologically different subpopulations within a heterogeneous mixture of cells. Simultaneously a series of reflecting filters separate the fluorescent emissions of each cell into specific wavelength categories, each of which is recorded by a different sensor. These sensors are called photomultiplier tubes (PMTs). In the Beckman flow cytometer used in this study 4 PMTs were present PMT2 (emission 525 nm), PMT3 (emission 575 nm), PMT4 (emission 610 nm) and PMT5 (emission 675 nm). An Argon laser was used as the light source (excitation 488 nm). PMT1 was the side scatter.

2.12 Cell cycle analysis method

2.12.1 Preparation of samples. MCF7 or MDA-MB 468 cells were pretreated with natural compounds or 0.1 % DMSO (negative control) for 24, 30 or 48 hrs. The medium was aspirated, the cells were washed with cold PBS, fixed in 70% ethanol and stored at least 2 hours in –20°C (Hughes and Mehmet, 2003). Alternatively cells can be fixed in 100% ethanol for 1 hr at –20°C, as described in Pan et al., 2002. The cells were then resuspended in propidium iodide solution (70 µg/ml in PBS) containing 13 Kunitz units of Rnase and incubated at 37°C for 30 minutes. Fluorescence was measured on a FACS Beckman flow cytometer.

2.12.2 FACS analysis

The machine was calibrated and the laser aligned using fluorescent beads and a flow check protocol, before each sample was run. All PMTs should give a CV of less than 2.5

in the calibration. All the parameters required for the DNA analysis protocol, which included Voltage, Peak gain, Integrated gain and Discriminator were optimised in preliminary experiments.

For PI PMT4 was used. A DNA histogram was obtained by plotting PMT4 peak (Y axis) against PMT4 integral (X axis). PMT4 peak corresponds to the voltage obtained by each particle, which is proportional to the maximum amount of fluoresence of each particle. PMT4 integral corresponds to the integral of the voltage, which is proportional to the total amount of fluoresence of each particle. Doublets were separated from single cells in G_2/M phase by gating. The data obtained from each histogram were then analysed using Multicycle Analysis 2.0 software and compared with manual estimation of the proportion of cells in each phase.

2.13 Preparation of RNA by guanidinium-acid-phenol extraction

The experiment was carried out as described by Chomczynski and Sacchi (Chomczynski and Sacchi, 1987). Briefly MCF-7 cells (at least 0.5 x 10^6 cells/ml) were lysed with 1ml of solution D (4M guanidinium thiocyanate, 25mM sodium citrate pH 7, 0.5% sarcosyl and 100 mM 2-mercaptoethanol) transferred to an Eppendorf tube and mixed with 0.1 ml of sodium acetate (2M pH 4.0), 1ml water saturated phenol and 0.2ml of chloroform: isoamyl alcohol (49:1). The sample was centrifuged at 4 °C for 20min at 10,000g and the aqueous phase (containing RNA) was removed and mixed with an equal volume of isopropanol. The sample was then stored at –20°C for 1 hr to precipitate RNA and further centrifuged at 4°C for 20min at 10,000g. The supernatant was removed and the resulting RNA pellet was mixed with 0.3ml of solution D and 0.3 ml of isopropanol and stored at –

20°C for 1 hr. The RNA was precipitated again with isopropanol with a last centrifugation step (4°C, 10,000g, 10 min), washed with 0.5ml of 75% ethanol and left to dry. 50µl of RNAse free water were then added to the tube and following quantitation (see 6.5.1) the RNA was stored at –80°C. This solution can be stored at these conditions for up to one year without appreciable deterioration.

2.14 Total RNA extraction with a binding column

MCF7 cells pretreated with natural compounds were trypsinised and transferred to a universal tube. Cells were centrifuged at 900 rpm for 5 minutes, the culture medium was removed and the pellet was lysed by addition of 250µl lysis solution (The solution was provided from SIGMA in the total RNA extraction kit and the exact composition of the washing, lysis solutions and filtration column remains unknown). The sample was vortexed thorougly to remove all clumps and the lysed cells were pipetted into a filtration column. The column was centrifuged for 2 minutes at 13,000 rpm. This step is essential to remove cellular debris. The filtration column was discarded and 250µl of RNAse free 70% ethanol was added to the filtered lysate, which was then transferred to a binding column. The column was centrifuged at 13,000 rpm for 15 seconds and the eluate discarded. 500µl of Wash solution 1 was added to the column and the column was centrifuged for the same time and at the same speed as above. Both the collection tube and the eluent were discarded and the column was transferred to a new collection tube with the above step repeated with Wash solution 2. Finally, a second 500 µl volume of Wash solution 2 was added to the column, which was dried under centrifugation at 13,000 rpm for 2 minutes. The flow through liquid was discarded and total RNA was

eluted by addition of 50 µl of elution solution and subsequent centrifugation at 13,000 rpm for 1 minute.

2.15 RNA gel electrophoresis

A 50 ml solution of 1xTAE (Sambrook and Russel, 2001)(Tris-Acetate-EDTA) buffer was prepared containing 1% agar. The solution was heated in the microwave for 2 minutes with continuos shaking to disolve the agar and then left to cool down at room temperature for 20 minutes. SDS was added at a final concentration of 0.1% and Ethidium Bromide at a final concentration of 0.2 µg/ml. The electrophoresis apparatus was rinsed with 3% H_2O_2 to eliminate any Rnase activity and the gel was poured in the electrophoresis chamber and left to set. Electrophoresis buffer (1XTAE) was added so that to cover the surface of the gel. The RNA samples were heated for 15 minutes at 65°C, to denature any secondary structure. The samples were loaded in the sample wells. Typically 5 µl of RNA sample was mixed with 10 µl of loading buffer, which contained 90% formamide (v/v), 0.25% xylene cyanol (w/v), 0.25% bromophenol blue (w/v) and 10% 1xTBE (Sambrook and Russel, 2001) (Tris-Borate-EDTA) (v/v). The gel was left to run for 2 hrs at 80Volts.

2.16 DNA gel electrophoresis

1%(w/v) agarose (0.5 g in 50 ml) was dissolved in 1 X TAE (Tris-Acetate-EDTA) and heated in the microwave. The solution was left to cool down for about 10-15 minutes and then 2µl of EtBr (10mg/ml) were added. The gel was left to set with casting combs on the electrophoresis chamber for about 30 minutes. 1 X TAE buffer was added in the chamber until the buffer just covered the gel. The DNA was mixed with loading dye and loaded into the wells of the gel. Electrophoresis was run at 90 volts for 30-40 minutes and the gel visualised on a long wave UV light box.

2.17 Nucleic acid concentration estimation and quality control techniques

The concentration of each RNA or DNA sample was calculated from the absorbance at 260 nm. This value multiplied by the factor 44.19 provides the concentration of the sample in µg/ml. The purity of each sample with respect to protein contamination was estimated from the 260/280 ratio. A pure RNA sample has a ratio of close to 2 ± 0.05, whereas a pure DNA sample of 1.8 ± 0.05. Furthermore the absorbance spectrum between 240 nm and 320 nm of nucleic acids exhibits a characteristic bell shaped curve with a maximum at 260 nm. Any deviations from this characteristic pattern indicate the presence of impurities. For the RNA samples the best diagnostic test is RNA gel electrophoresis.

2.18 Reverse transcription

Enhanced Avian Reverse Transcriptase was purchased from Sigma and the protocol adapted from the information provided. RNA template of interest (final conc 0.005-0.25μg/μl) was mixed with deoxynucleotide mix (500 μM of each dNTP) and anchored oligo$(dT)_{23}$, cenrtifuged to collect all the reagents to the bottom of the tube and heated at 70°C for 10 minutes. The tube was placed on ice and Rnase inhibitor (1U/μl) along with 10X reverse transcriptase buffer were added. Reverse transcription was initiated with the addition of the enzyme (1U/μl of enhanced avian reverse transriptase) at 43°C at a final volume of 20μl. The incubation was extended for 50 minutes at this temperature. The first strand cDNA was stored at -80°C for subsequent PCR amplification.

2.19 Polymerase chain reaction

The method including primer sequences, reagent concentrations and cycling parameters was adapted from Dohr et al., 1995. PCR primers were purchased from Invitrogen (Paisley UK) predicting a fragment of 541, 146 and 360 base pairs for *β-actin* , *CYP1A1* and *CYP1B1* respectively. PCR reactions were carried out in a final volume of 50μl containing 3-4μl of cDNA, 5μl of 10X *Taq* buffer and 200 μM of each dNTP in the presence of 0.2 μM of each primer and 2.5 units *Taq* DNA polymerase. Amplifications were performed using a DNA thermal cycler for 25-30 cycles depending on the DNA sequence with the following profile: 4 min at 94°C for inactivation for the first cycle, 1min at 94°C for denaturation, 1 min for primer annealing, 1 min at 72°C for primer

extension and 7 min at 72°C for the last cycle for final extension. The primer nucleotide sequences are shown below:

CYP1A1

FP: TAGACACTGATCTGGCTGCAG

RP: GGGAAGGCTCCATCAGCATC

CYP1B1

FP: AACGTCATGAGTGCCGTGTGT

RP: GGCCGGTACGTTCTCCAAATC

β-Actin

FP: GTGGGGCGCCCCAGGCACCA

RP: CTCCTTAATGTCACGCACGATTTC

2.20 CYP1 family enzyme activity in intact MCF7 cells

For assays of EROD activity in intact cells, cells in 24-well plates were treated with compound for the time and concentration indicated. The EROD activity was measured as described by Kennedy and Jones, 1994. The medium was aspirated and the cells were washed with PBS (usually 2ml in each well). 270µl of fresh medium containing 1.5 mM salicylamide to inhibit conjugating enzymes, were added to the wells. The plate was left in the incubator at 37°C for 5 minutes and 30µl medium of ethoxyresorufin (50µM) were added to give 5µM as a final concentration. The plate was left in the incubator at 37°C for 1 hr with gentle stiring every 5 minutes. 200µl medium was transferred in ependorf tubes and the reaction was terminated with the addition of 200µl of ice-cold methanol.

The samples were centrifuged at 3,000 rpm for 10 minutes and the supernatant was transferred to a 96 well plate and read using a fluoresence plate reader with excitation and emission at 530 and 590nm respectively. Standard curves for resorufin formation were also performed.

2.21 Statistical analysis

Experiments were repeated at least two times. Significant differences for n=3 or n=4 replicates were determined by Student's T test. Statistical analyses were performed using SPSS.

3.FLAVONOIDS AS INHIBITORS OF CYP1 ENZYMES

3.1 Introduction

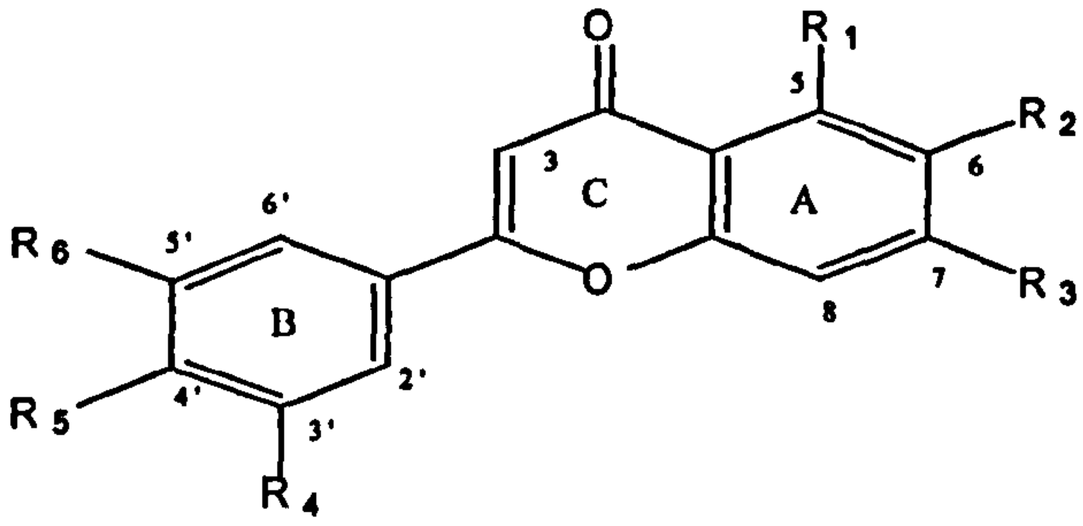
The long known preventative effect of plant based diets on carcinogenesis and related chronic diseases is well documented and the flavonoids have been indicated as cancer preventative compounds by numerous epidemiological, *in vitro* and *in vivo* studies as described in detail in the introduction chapter. Amongst the different mechanisms of action, which explain the chemopreventative nature of the flavonoids, their interaction with cytochrome P450s has also been proposed (Middleton et al., 2000). This type of interaction will have a significant impact in the case of CYP1 enzymes, since they are involved in the metabolsim of PAHs and related compounds, as described in section 1.3.3 and therefore may provide more evidence in the chemopreventative function of dietary flavonoids. Doodstar et al. (2000) has shown that flavonoids can inhibit CYP1 enzymes. Thus, there is great interest to identify which flavonoids interact with the CYP1 family enzymes and whether that interaction is inhibition of their activity or a different mechanism of action. The purpose of the work described in this chapter was to examine a group of structurally related flavonoids, through their ability to inhibit CYP1 enzymes. We used the EROD assay described in section 2.3 to screen a panel of naturally occuring flavonoids, in terms of inhibitory potency (IC_{50}) towards CYP1 family enzymes.

3.2 Results and discussion

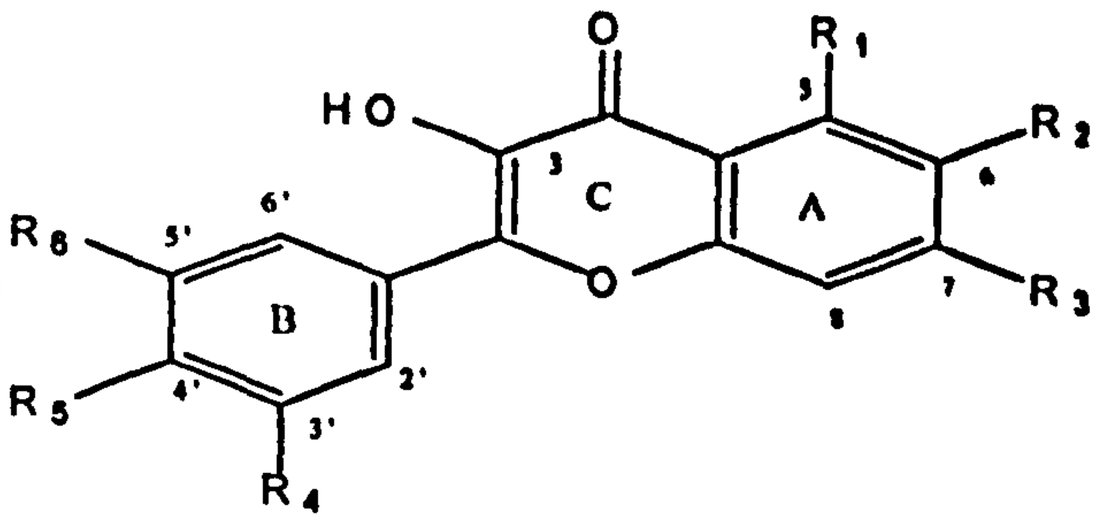
The inhibitory effects of flavonols and flavones on CYP1 supersomes are reported in Table 2, while the different structural components of each compound are summarised in Table 1. All the compounds except eupatorin-5-methyl ether, cirsiliol and genkwanin inhibited CYP1B1 activity more effectively than they did CYP1A1 and CYP1A2 activity. The flavone chrysin was the most potent inhibitor of CYP1A1 and CYP1A2. Diosmetin and chrysin also inhibited CYP1B1 at lower concentrations compared to all the other compounds (IC_{50} 0.5 μM). The flavonols quercetin, myricetin and kaempferol were also potent inhibitors of CYP1B1 activity, whereas they seemed to be less effective for CYP1A1 and CYP1A2, with the exception of the latter showing more activity for CYP1A1. The flavones genkwanin, cirsiliol and eupatorin-5-methyl ether were very weak inhibitors of CYP1 enzymes. 6-OH luteolin and baicalein also were very weak for CYP1A1 and CYP1A2 and slightly more potent for CYP1B1. Apigenin, luteolin and eupatorin were of intermediate efficacies. Some of the compounds tested seemed to possess selectivity against a certain isoform, such as luteolin, 6 hydroxy luteolin, baicalein, kaempferol, quercetin, myricetin for CYP1B1 and eupatorin-5-methyl ether for CYP1A1. The flavonols had the highest selectivity for CYP1B1 compared to CYP1A1 (up to 9 fold) and CYP1A2 (up to 13 fold).

In the present study evidence is given that dietary flavonoids can inhibit CYP1 enzymes. A novel synthetic chalcone (DMU 713, Tan, 2005) was used as a positive control (data not shown). The flavone chrysin and the flavonols quercetin, myricetin and kaempferol were potent inhibitors of CYP1A1 and CYP1B1 activity. Flavonoids with hydroxy substitutions at positions 5 and 7, such as chrysin and luteolin are potent inhibitors of

CYP1 enzymes. These substitutions are possibly required for docking the flavone molecule to the active sites of the CYP1 enzymes. Hence 6 hydroxy substitution decreased the inhibitory activity and methoxy substitution at position 7 completely abolished it. In the case of diosmetin the 4'-methoxy substitution seems to have a profound effect on CYP1A1 and



Flavones



Flavonols

Compound Class	R_1	R_2	R_3	R_4	R_5	R_6
Flavones						
Chrysin	OH	H	OH	H	H	H
Baicalein	OH	OH	OH	H	H	H
Apigenin	OH	H	OH	H	OH	H
Luteolin	OH	H	OH	OH	OH	H
6OH Luteolin	OH	OH	OH	OH	OH	H
Diosmetin	OH	H	OH	OH	OMe	H
Eupatorin	OH	OMe	OMe	OH	OMe	H
Cirsiliol	OH	OMe	OMe	OH	OH	H
Genkwanin	OH	H	OMe	H	OH	H
Eupatorin-5-methyl ether	Ome	OMe	OMe	OH	OMe	H
Flavonols						
Kaempferol	OH	H	OH	H	OH	H
Quercetin	OH	H	OH	OH	OH	H
Myricetin	OH	H	OH	OH	OH	OH

Figure 3.1 Structural features of different classes of dietary flavonoids

CYP1B1 inhibitory activity as compared to luteolin. Moreover eupatorin having a 4' methoxy group and a 5 hydroxy group was a lot more potent CYP1B1 and CYP1A1

inhibitor than eupatorin-5-methyl ether. Overall flavonols were potent inhibitors towards CYP1B1 activity, compared to their flavone equivalents, indicating that the hydroxy group at position 3 of the C ring increases inhibitory activity. This was not the case for CYP1A1.

Table 3.1 The effect of dietary and synthetic (6-OH luteolin) flavonoids on the EROD activity of human CYP1 enzymes. CYP1 (CYP1A1, CYP1A2, CYP1B1) microsomes (5 pmol/ml) were incubated with various concentrations of flavonoids (25, 2.5, 0.25, 0.025, 0.0025 μM). Results are expressed as IC_{50s}, calculated from percentage of inhibition of EROD activity compared to control. Each value corresponds to mean ± standard deviation for n = 4 determinations. IC_{50s} are in μM.

Compound	CYP1B1	CYP1A1	CYP1A2
Quercetin	0.7 ± 0.3	7 ± 0	12 ± 3.5
Myricetin	0.9 ± 0.2	4 ± 0	13 ± 0.7
Kaempferol	1.3 ± 0.2	8 ± 1.4	9 ± 2.1
Apigenin	2 ± 0	4.2 ± 0	6 ± 0
Luteolin	1.8 ± 0.4	10 ± 0.7	15 ± 1.4
6OH Luteolin	7.7 ± 2	>25	>25
Diosmetin	0.5 ± 0.1	1.2 ± 0.3	18 ± 4
Baicalein	7 ± 1.1	20 ± 0	20 ± 0
Chrysin	0.5 ± 0.1	0.5 ± 0.1	2 ± 0
Eupatorin	1.1 ± 0.1	2.3 ± 0.1	25 ± 0
Eupatorin-5-methyl ether	21 ± 1	6.8 ± 0.4	>25
Genkwanin	>25	>25	>25
Cirsiliol	>25	>25	>25

The above results provide further insight to previous studies, which underlined the importance of A and B ring substituents in the flavonoid structure for inhibition of CYP1 enzymes. Therefore diosmetin and acacetin, which lack the 3′-hydroxy group, have already been reported to be a potent inhibitors of CYP1B1 and CYP1A1 activity

(Doodstar et al., 2000). Zhai and coworkers (1998) has also shown that the hydroxy substitutions at positions 3 and 7 of the flavones are important for inhibition of CYP1A1 enzyme and hydroxy substitutions at positions 3 and 5 increase the inhibition towards CYP1A2, which is in concordance with the data reported here. Furthermore, Tsyrlov et al. (1994) suggested that 3′ and 4′ positions have a profound effect on the inhibitory action of the flavonoids, which does not seem to be necessarily the case, since chrysin which does not contain any substituents on the A ring was the most potent inhibitor for all three CYP1 enzymes.

Selective inhibitors of CYP1B1 and CYP1A1 have already been identified (Chun et al., 2001, Chun et al., 2002). Although they are natural products they do not belong in the flavone subfamily. Rhapontigenin is a selective CYP1A1 inhibitor (Chun et al., 2001) with an IC_{50} of 0.4 μM. Surprisingly the structure of this molecule can be overlayed on diosmetins structure. Both of them contain the same substituents in the A and B rings. The only difference is that rhapontigenin is a stilbene and diosmetin is a flavone. The data presented here show that diosmetin is a potent inhibitor of both CYP1A1 and CYP1B1 but not of CYP1A2. Hence, selectivity towards one CYP1 family enzyme is lost, due to the six carbon membered ring which is present in the flavone structure. Similarly the structure of resveratrol can be overlayed on apigenin. The inhibitory potency of apigenin towards CYP1 family enzymes followed the order: CYP1B1 > CYP1A1 > CYP1A2. Resveratrol has been reported to inhibit CYP1A1 enzyme activity in cell based assays (Ciolino and Yeh, 1999) as well as enzyme assays with microsomes prepared from recombinant human P450 CYP1A1 and CYP1A2 (Chun et al., 1999). The authors of this study showed that resveratrol had an IC_{50} of 23 and 1200 μM against

CYP1A1 and CYP1A2 respectively, as reported by EROD activity, thus being 50 fold more selective for CYP1A1. The results here show that apigenin is a more potent inhibitor than resveratrol against all three CYP1 enzymes. Tetramethoxystilbene has been reported to be a selective CYP1B1 inhibitor (Chun et al., 2002). Unfortunately none of the compounds tested resembled that structure and therefore no conclusions can be drawn, regarding structure activity relationships.

Although most of the previous studies have concentrated on inhibition of CYP1 family enzymes by natural products or related synthetic analogues, it is important to note that selective CYP1B1 and CYP1A enzyme expression in tumour cells, compared to normal cells, is a very attractive target for chemotherapy and chemoprevention via prodrug activation. The identification of a selective CYP1B1 substrate can have great therapeutic implications in the treatment of cancer, because it will not get metabolised by normal cells. The flavonoids which were shown to be potent inhibitors in this study, could very well be potential substrates. Resveratrol as mentioned above has been reported to be a substrate and an inhibitor. The EROD assay does not differentiate between the different types of inhibition. A molecule which is a strong competitive inhibitor, could be a very good substrate, since it will fit in the active site of the enzymes. Further work in chapter 4 decribes the substrate specificity of such dietary constituents for CYP1 family enzymes.

4. THE METABOLISM OF NATURAL FLAVONES BY CYP1 FAMILY ENZYMES

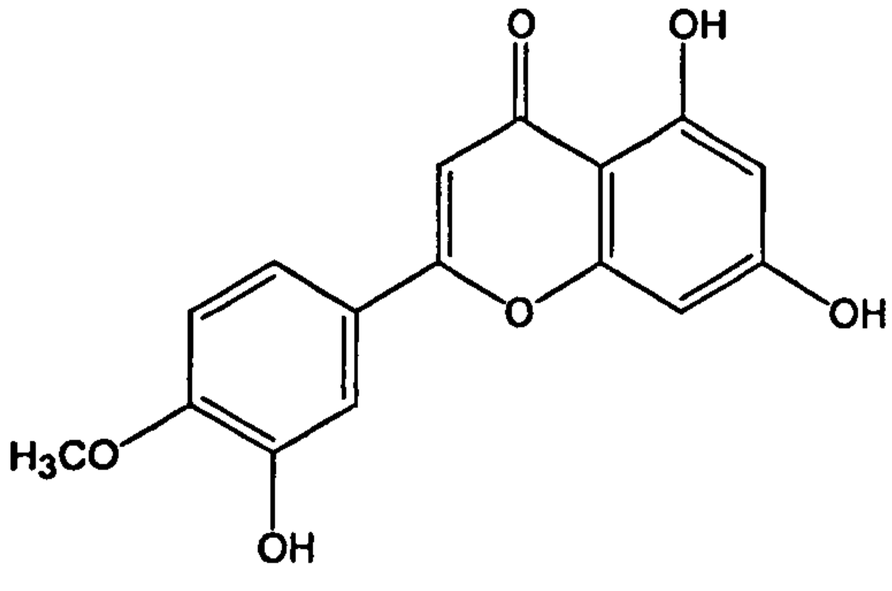
4.1 Introduction

The ability of the enzymes CYP1B1, CYP1A1 and CYP1A2 to metabolise PAHs and oestradiol is well documented in *in vitro* enzyme and cell based assays(Larsen et al. 1998, Kristensen and Borresen-Dale, 2000, Badawi et al., 2001). However little is known about the metabolism of natural products from such enzymes. To date the only study, which has shown metabolism of natural products from CYP1B1 was conducted in our laboratory. The natural product investigated was the stilbene resveratrol. Resveratrol is converted to piceattanol by CYP1B1 and to two other metabolites (Potter et al, 2002). One of them is probably produced from 4-hydroxylation of the second aromatic ring, as the authors suggested. Flavones have very similar structure to stilbenes. The main differences are the keto group at C-4 position and the oxygen atom at position 1, which are absent from stilbenes and present in the C ring of the flavones. The structures of oestradiol, flavones and stilbenes could be overlayed on each other. Thus it was hypothesized that CYP1 family enzymes will hydroxylate unsubstituted positions of the aromatic rings of the flavones. In order to justify this hypothesis metabolism studies of the flavones with CYP1 family enzymes were performed. A similar enzymatic assay as the one described previously (Potter et al., 2002) was used. Apigenin and luteolin were investigated from previous students and found to be substrates for the CYP1 enzymes and CYP1B1 respectively, whereas the flavonols quercetin and kaempferol were poor substrates for CYP1 enzymes (Wilsher, 2003).

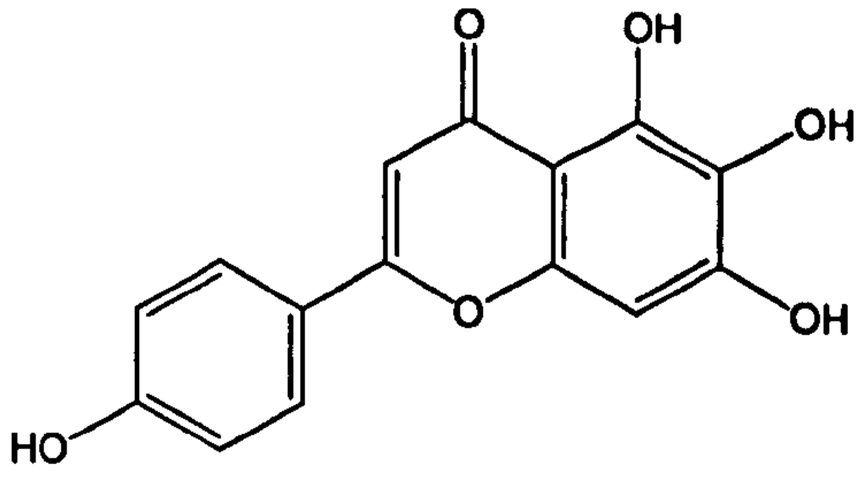
A few methoxylated flavones were also chosen in these studies, in order to to test whether CYP1 enzymes can catalyse demethylation reactions in this type of flavonoid subfamily. CYP1 enzymes catalyse the deethylation of 7-ethoxyresorufin to resorufin

(Burke et al., 1994). Furthermore it has been shown that hersperitin (the flavanone equivalent of diosmetin) is converted to eriodictyol (the flavanone equivalent of lutcolin) by CYP1A2 and human liver microsomes (Breinholt et al., 2002). Figure 4.1 shows the structures of the flavones, which were in investigated in the experiments described in this chapter.

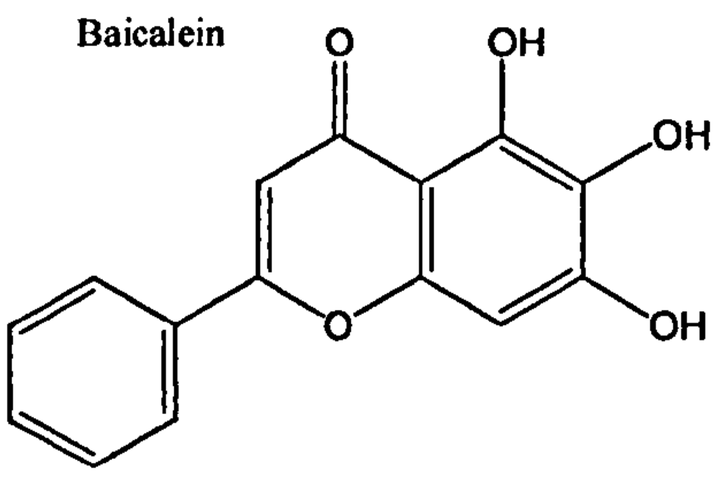
Diosmetin



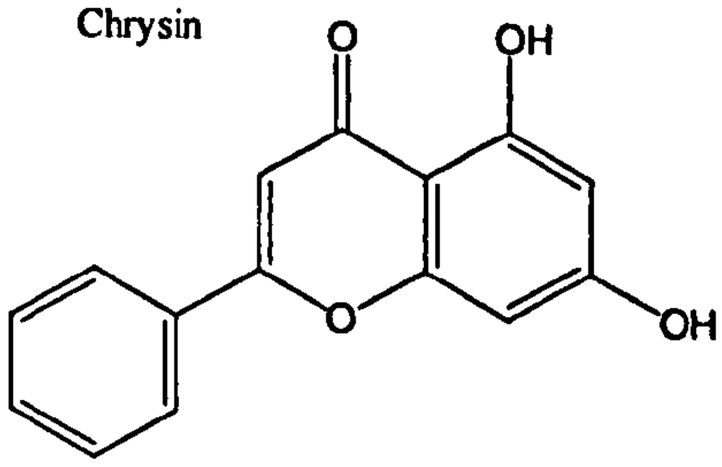
Scutellarein



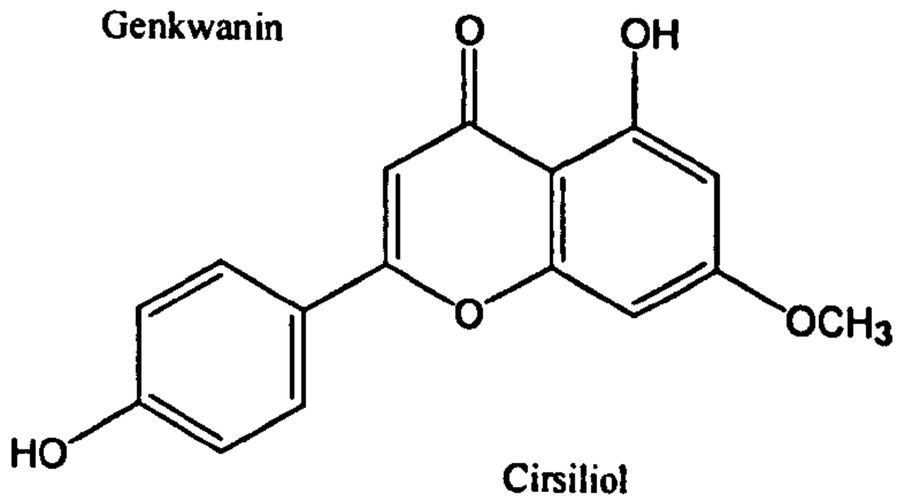
Baicalein



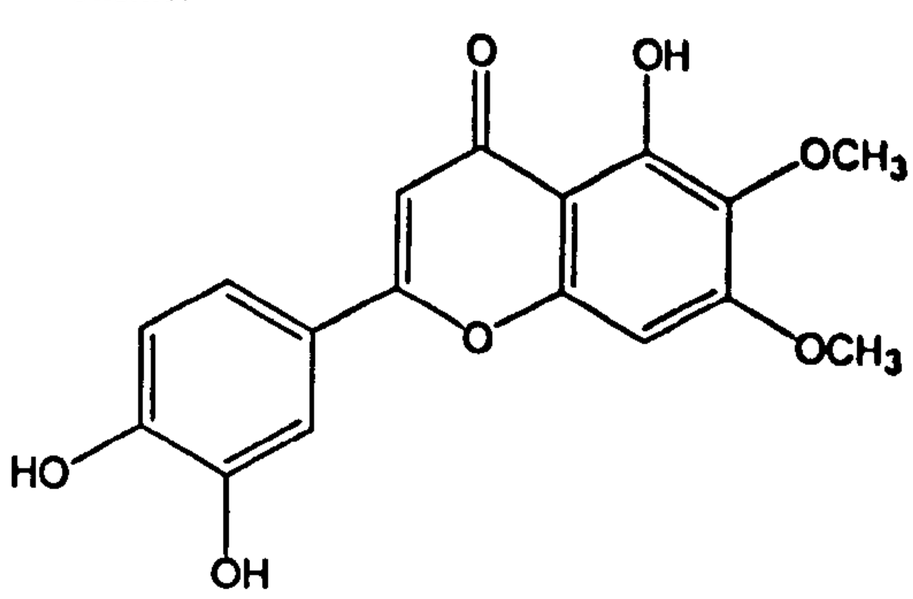
Chrysin



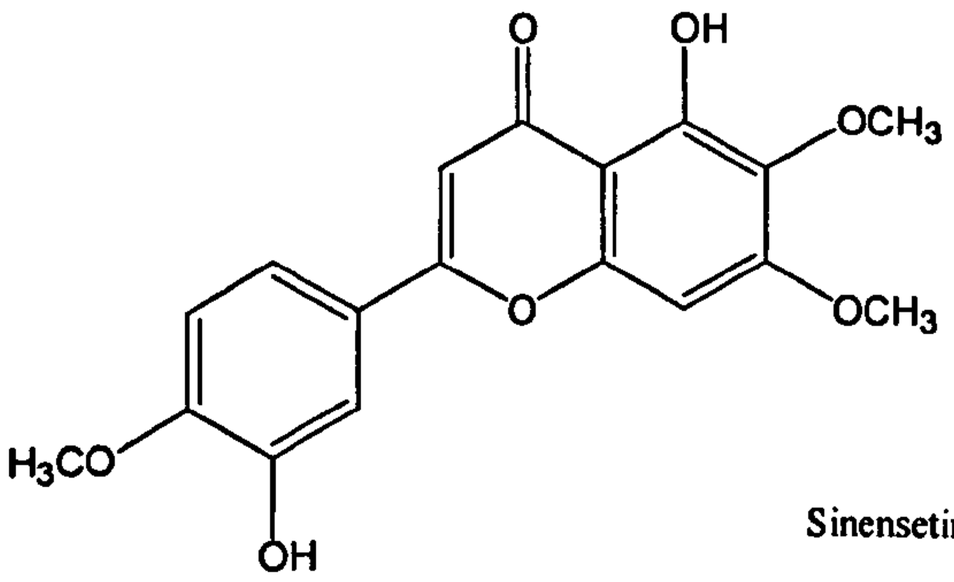
Genkwanin



Cirsiliol



Eupatorin



Sinensetin

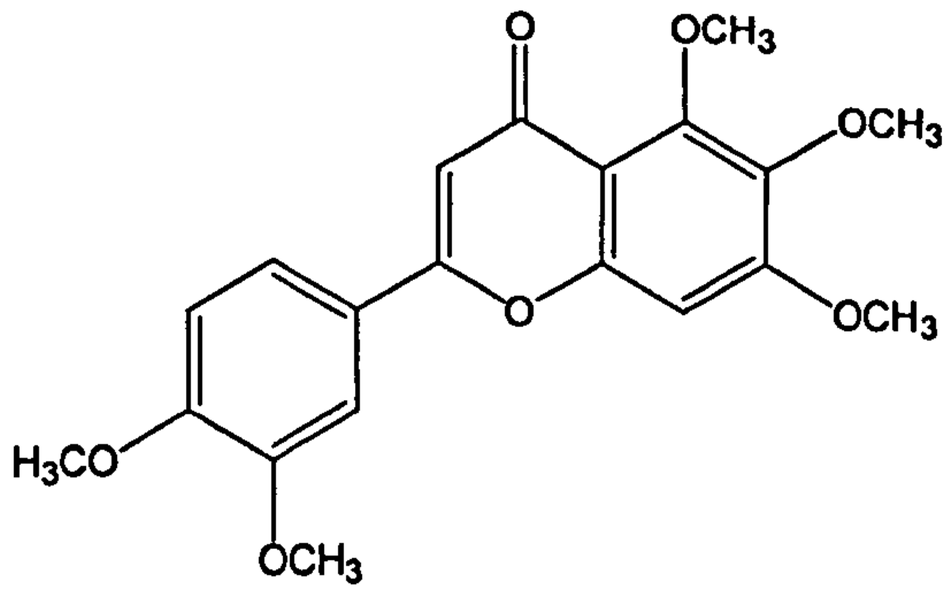
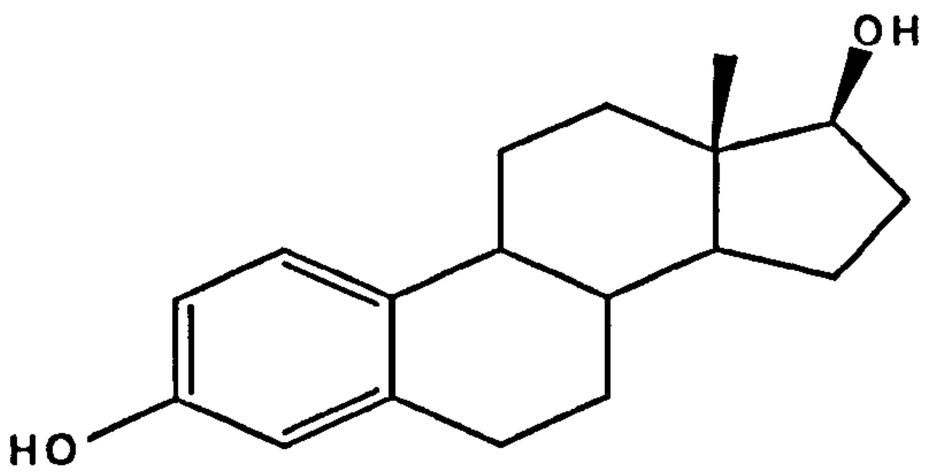
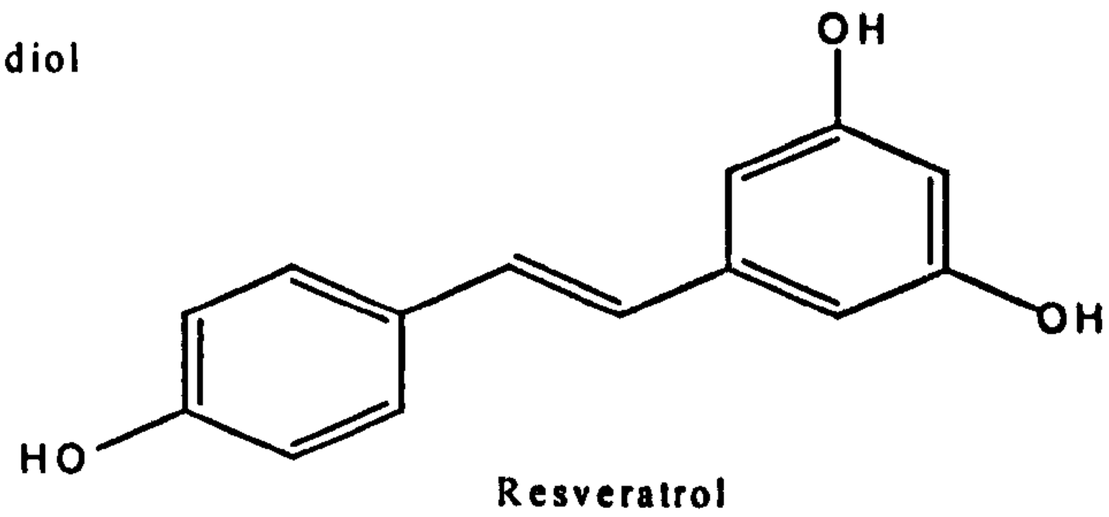


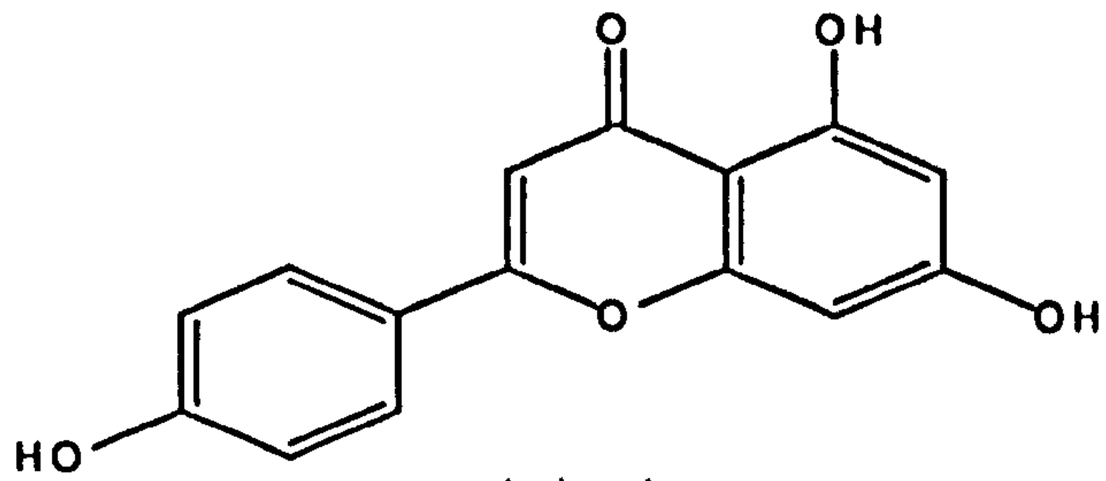
Figure 4.1 Structures of dietary flavones



Oestradiol



Resveratrol

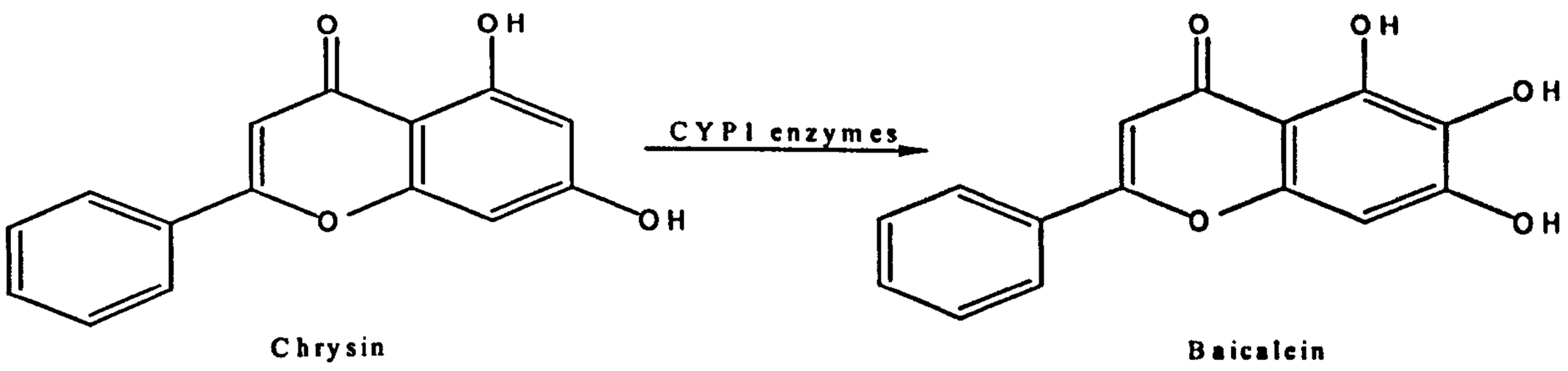


Apigenin

Figure 4.2 2D Mapping of oestradiol, resveratrol and apigenin, a naturally occuring flavone

4.2 Results and discussion

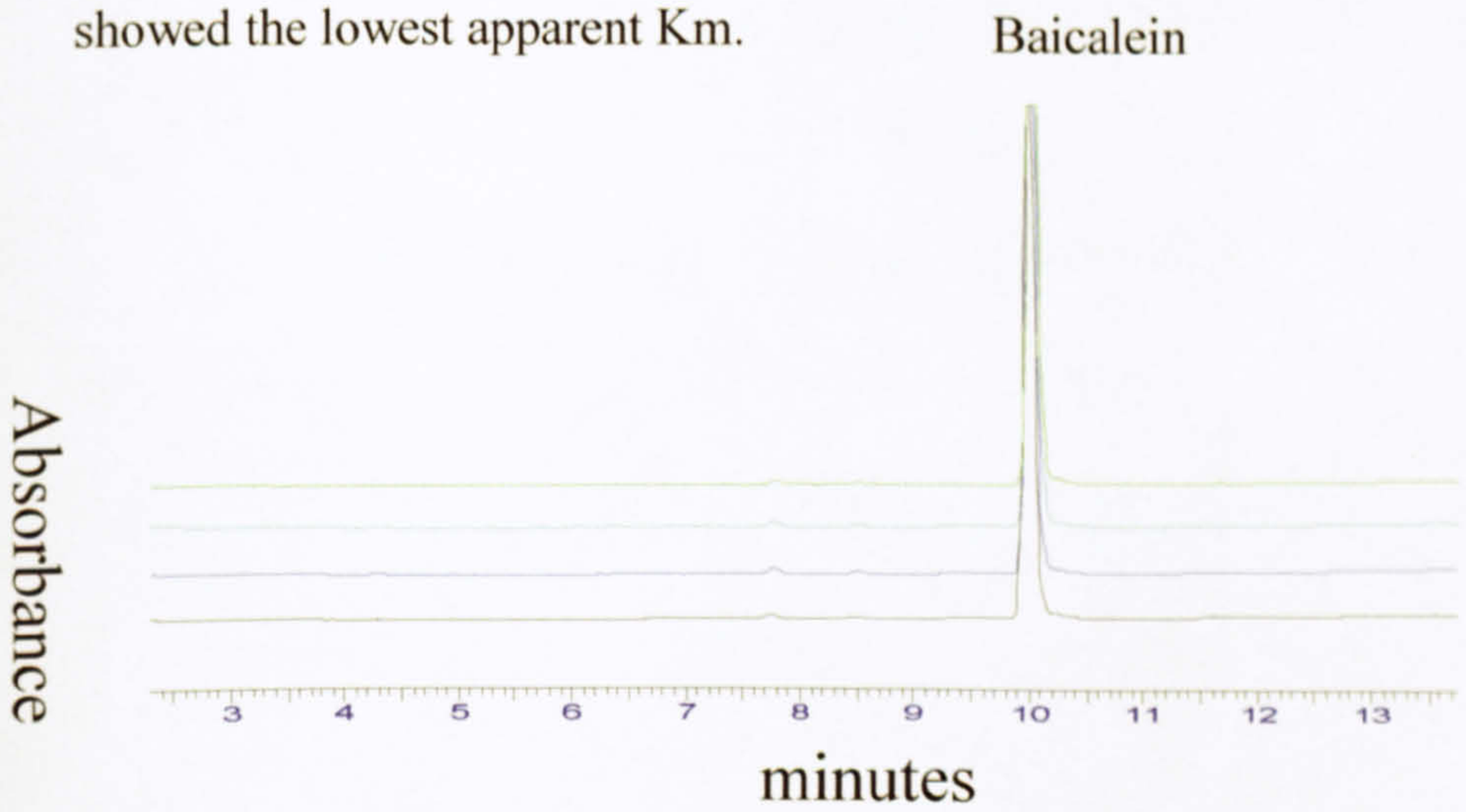
4.2.1 Metabolism of hydroxylated flavones



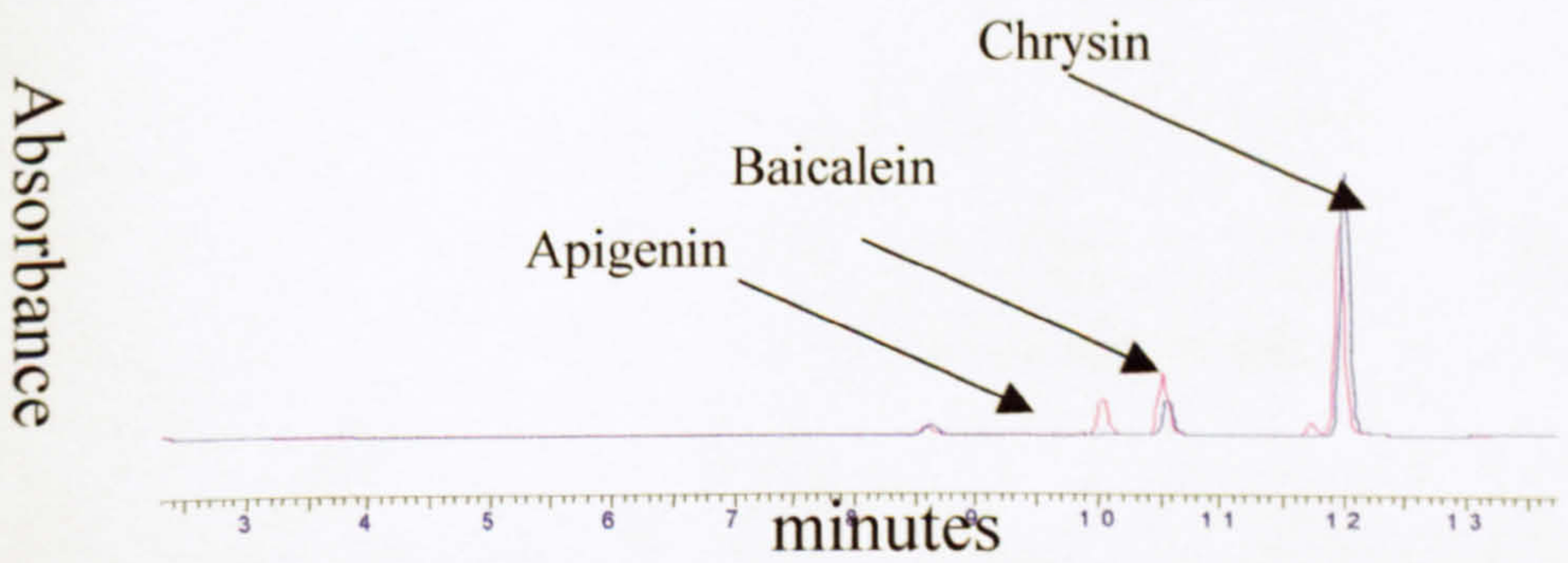
Chrysin Baicalein

Figure 4.3 The conversion of chrysin to baicalein

Incubation of baicalein with CYP1 family enzymes did not show any metabolism (Figure 4.4a). Chrysin was metabolised exclusively to baicalein by all three enzymes, as shown in Figure 4.4b. The kinetics of the reaction were investigated and the results are presented in Figure 4.5. CYP1A1 produced more baicalein than the other two enzymes and CYP1A2 showed the lowest apparent Km.



a) Baicalein; Black control, blue CYP1A1, green CYP1B1, light green CYP1A2



b) Chrysin. The sample was spiked with baicalein and apigenin authentic standard(1 µM); Pink standards, blue incubate

Figure 4.4 HPLC chromatograms of 20 min incubation of CYP1 enzymes with a) baicalein (10 µM)and b) chrysin (10 µM).

The difference in baicalein retention time between the two chromatograms, is due to method optimisation. The column C18 Luna 4.6 x 150 mm 5µ was replaced with a Polar-RP 4µ 150 x 4.60 mm and this improved chromatographic analysis. Baseline separation was achieved for apigenin and baicalein and this resulted to slightly greater retention time compared to the previous method. All the other conditions were left the same.

Parameter	Enzyme		
	CYP1B1	CYP1A1	CYP1A2
Km (µM)	0.36	2.9	0.31
Kcat (pmol/min/pmol of enzyme)	2.3	11.6	2.3

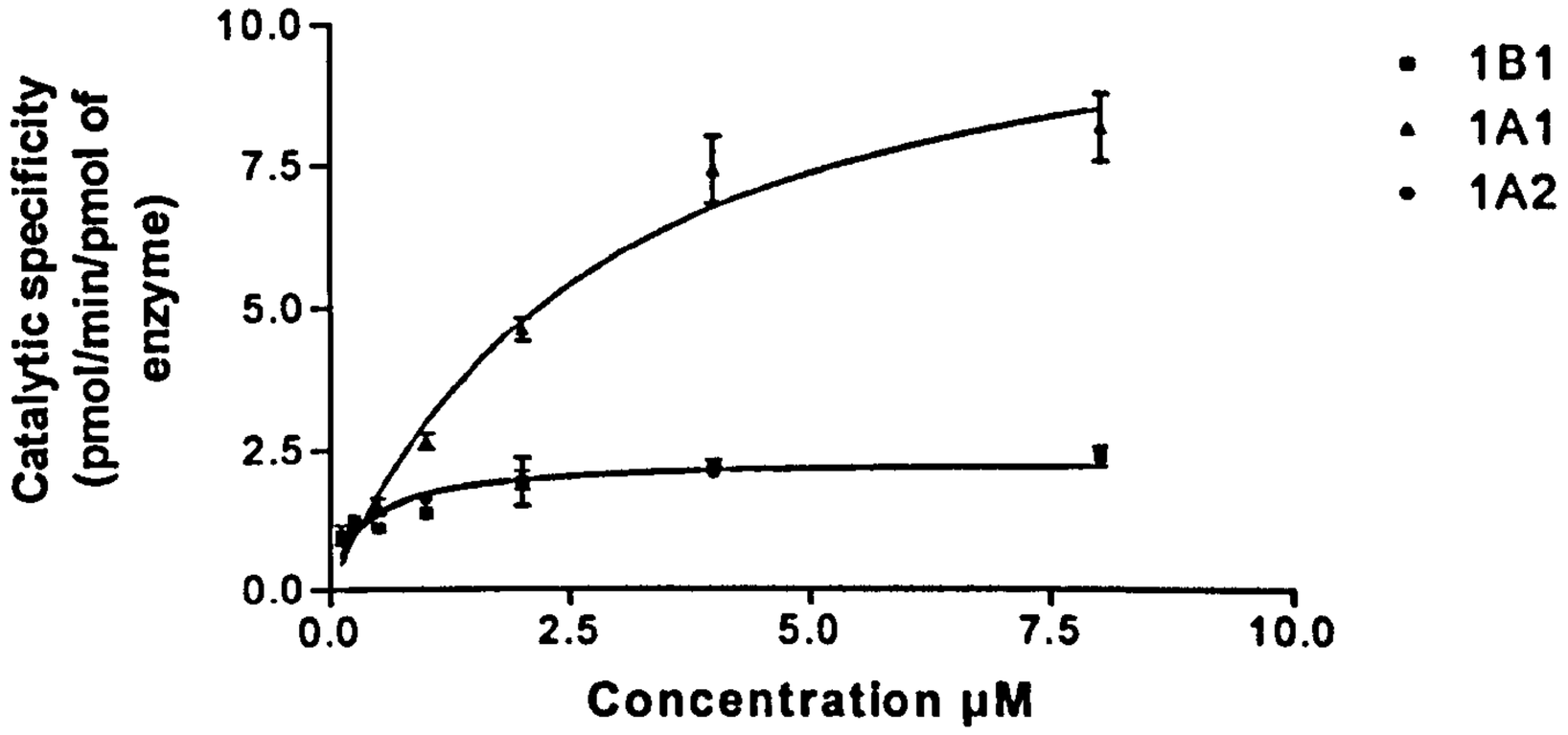
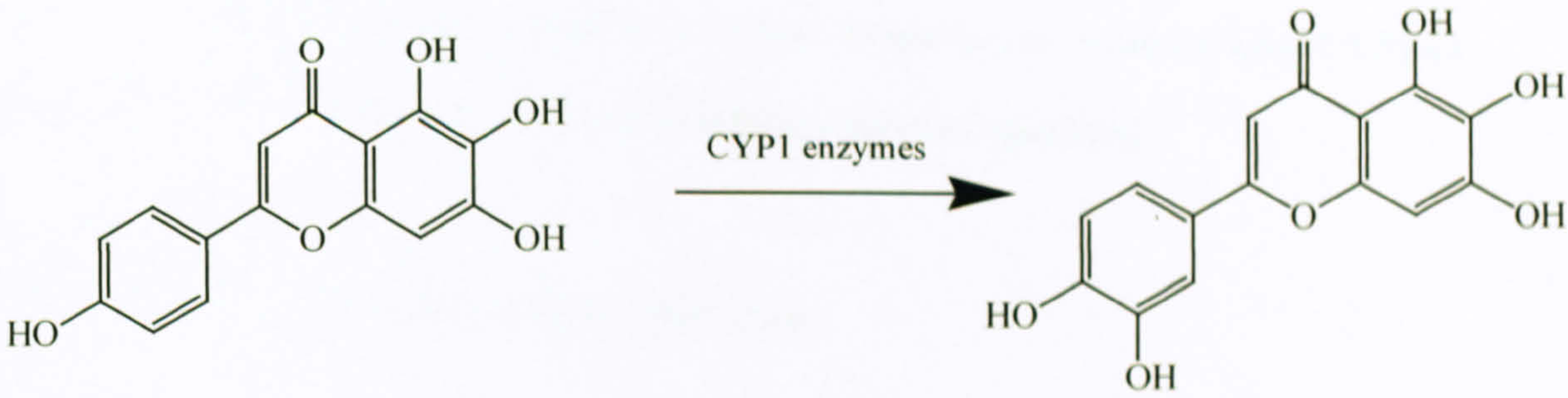


Figure 4.5 Enzymatic mechanism and determination of kinetic parameters of chrysin 6 hydroxylation. Error bars represent min and max values of n=2 determinations.



Scutellarein 6-hydroxyluteolin

Figure 4.6 The conversion of scutellarein to 6-hydroxyluteolin

Scutellarein (6-hydroxyapigenin) metabolism from CYP1 isozymes yielded a metabolite more polar than the parent compound, which was identified by coelution studies to be 6-hydroxyluteolin. This compound was formed to a smaller extent from CYP1B1, compared to CYP1A1 and CYP1A2. Other peaks shown in the chromatograms (Figure 4.7) are impurities from the parent compound.

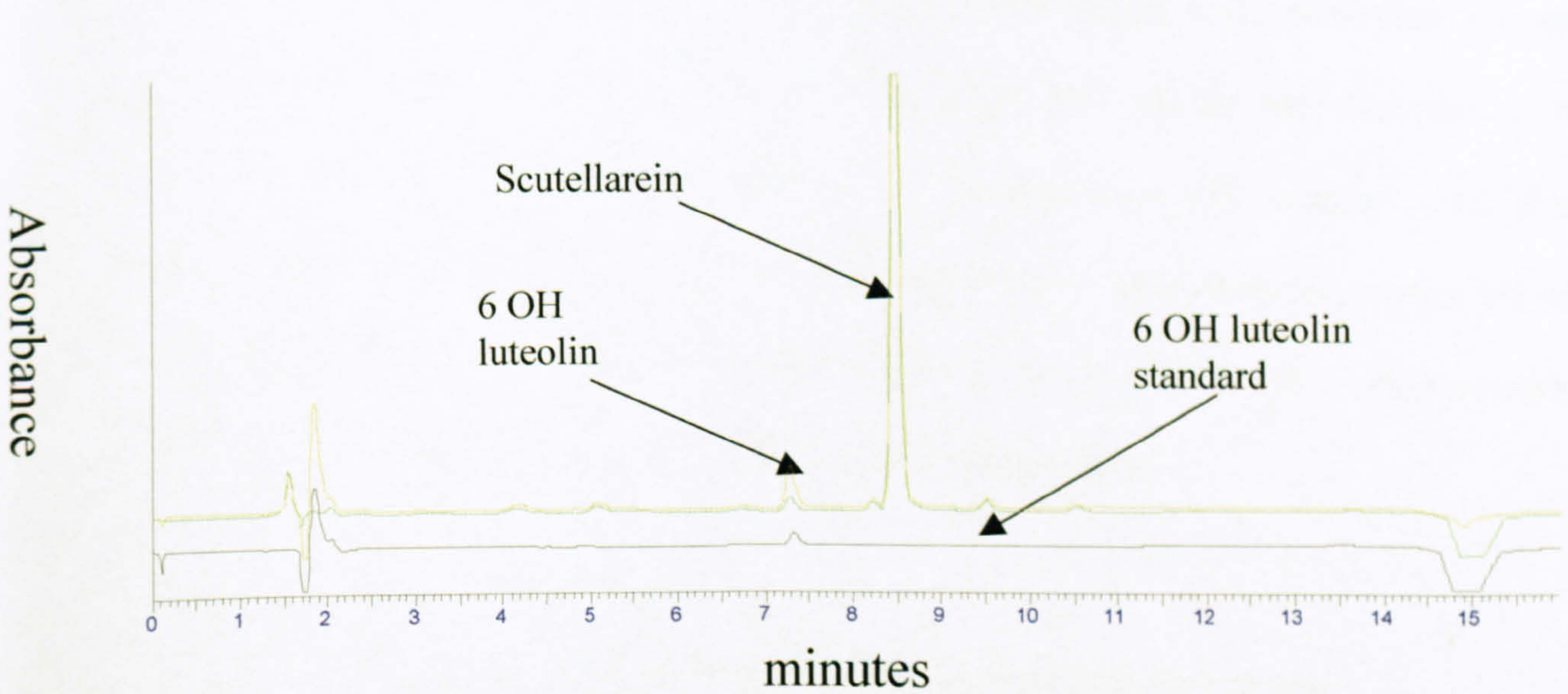
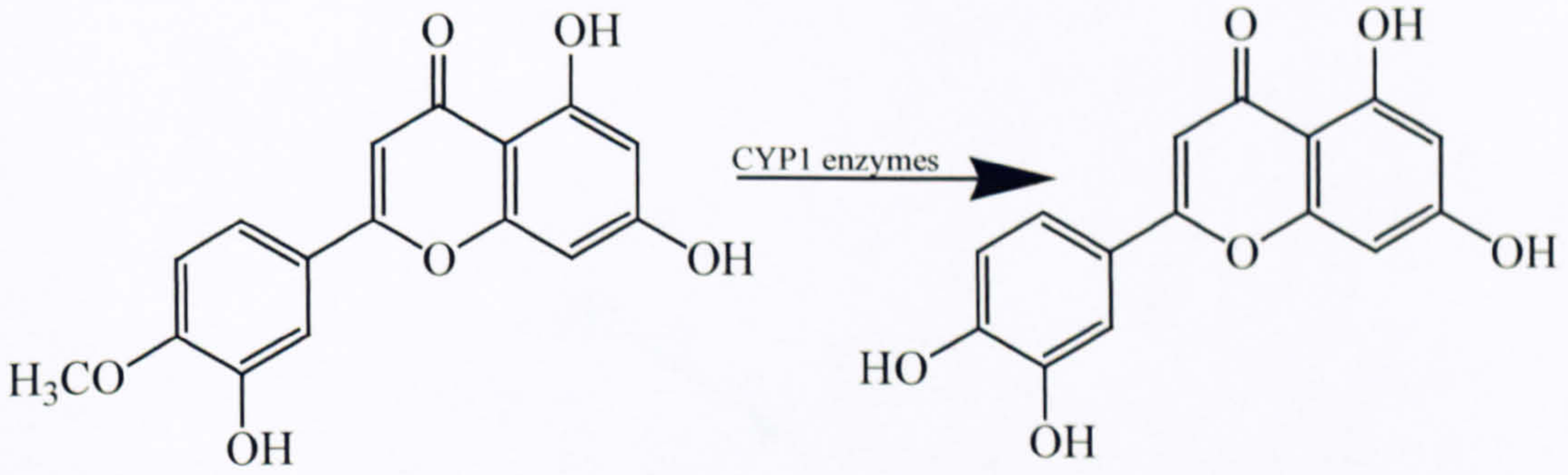


Figure 4.7. Coelution studies of scutellarein (10 μM) incubation samples from CYP1A1 enzyme with 6 OH luteolin authentic standard solutions (0.5 μM). The same coelution pattern was observed for the two other enzymes. Experiments were done in duplicate. Black

trace: 6 hydroxy luteolin standard, light green trace: Scutellarein incubate with CYP1A1, dark green trace: Scutellarein incubate and 6 hydroxy luteolin standard.

4.2.2 Metabolism of methoxylated flavones



Diosmetin Luteolin

Figure 4.8 The metabolism of diosmetin to luteolin

Diosmetin was metabolised mainly to luteolin (Figure 4.8 and 4.11) by all three enzymes but mostly from CYP1A1 and CYP1A2. Figure 4.9 shows the decrease in the concentration of the compound following incubation by CYP1 enzymes over time, compared to control experiments. Two other metabolites were observed, named D1 and D2, which seemed to be more polar than luteolin, as shown by the HPLC chromatogram (Figure 4.10) and hence were eluted at smaller retention times.

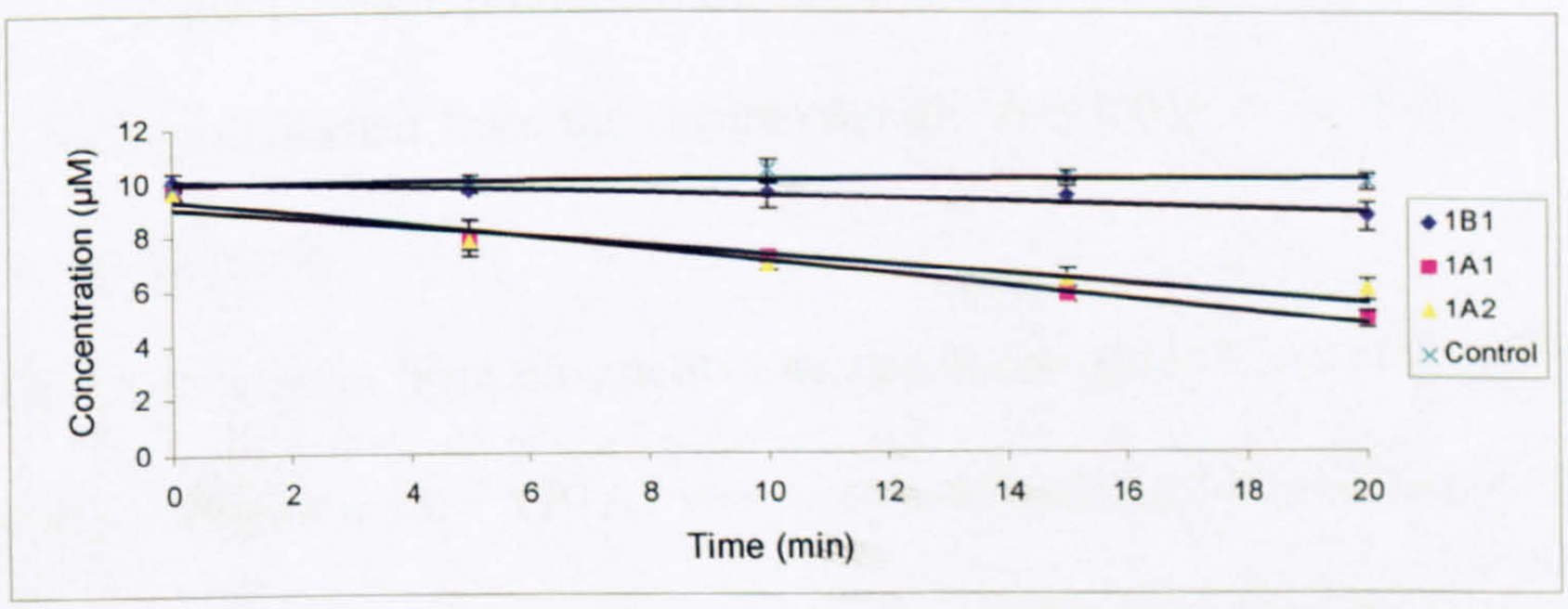


Figure 4.9 Rate of metabolism of diosmetin from CYP1 isozymes. Error bars represent tthree independent experiments.

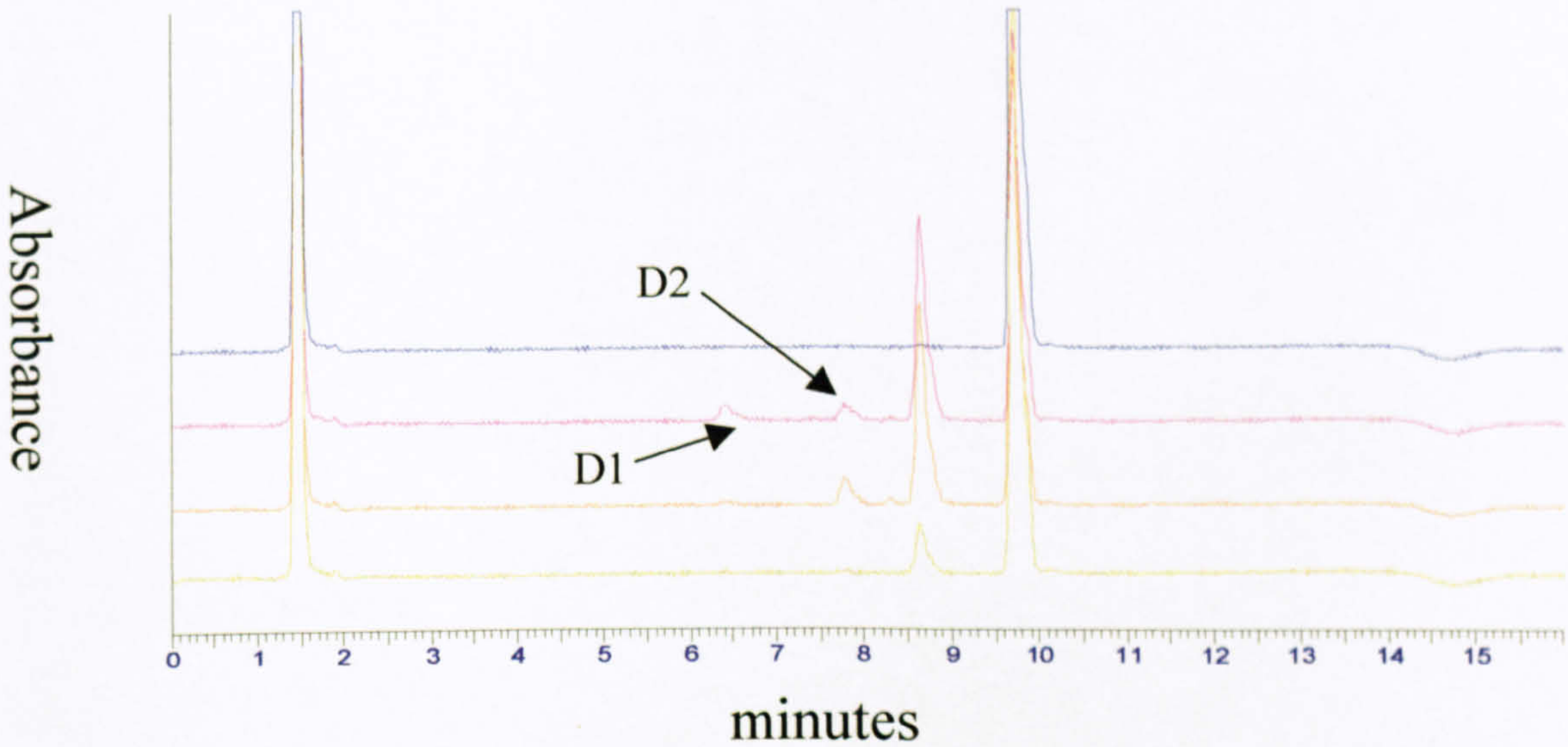


Figure 4.10 Metabolites formed after 20 min incubation of diosmetin (10 μM) with CYP1 family enzymes. Blue; control, pink; CYP1A2, orange; CYP1A1, dark green; CYP1B1.

D1 was identified as 6 hydroxy luteolin (Figure 4.12). 6-hydroxyluteolin was only a minor product from CYP1A2 and CYP1A1 mediated metabolism, whereas in the case of CYP1B1 no D1 and D2 were produced. D2 could not be identified, however according to the retention time indicated from the chromatogram, it is likely to be the 6 hydroxylated metabolite of diosmetin.

The formation of luteolin from diosmetin was also investigated kinetically and the results are presented in Figure 4.13. CYP1A1 was more efficient in promoting the demethylation at 4′ position of the B ring compared to the other two enzymes. Finally the metabolism of

diosmetin was also investigated in a hepatic CYP panel (Figure 4.14). There was no major decrease in the concentration of diosmetin within 20 minutes incubation with various CYPs (CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A5).

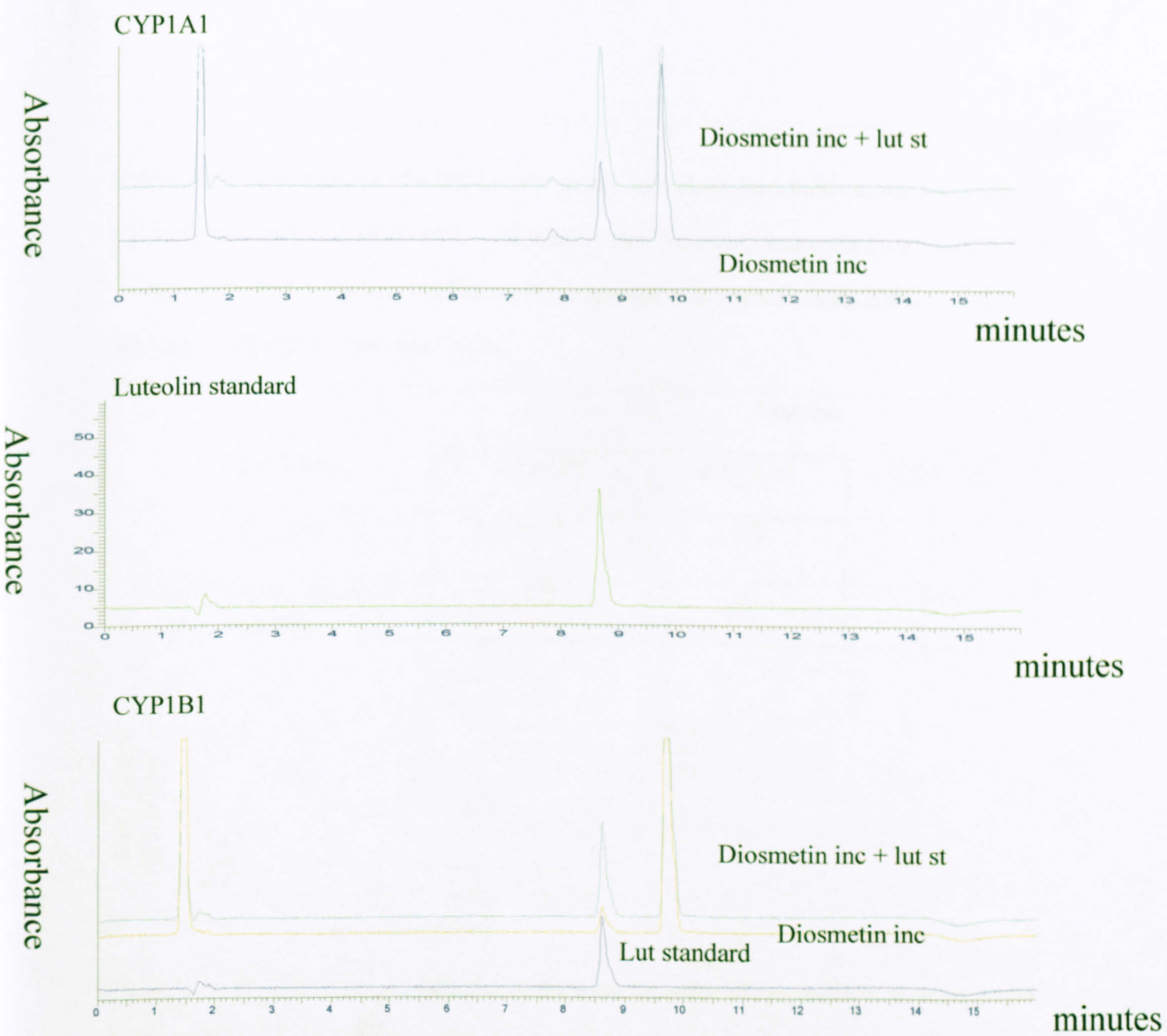


Figure 4.11 Identification of luteolin as the primary metabolite of the metabolism of diosmetin (10 μM) from CYP1 family enzymes. A 20 min CYP1A1 or CYP1B1 incubate was

spiked with 2μM of luteolin. Similar profile was obtained for CYP1A2 (data not shown). Experiments were done in triplicate. Luteolin standard has been added in the traces for clarity.

Figure 4.12 Identification of 6 OH luteolin as the secondary metabolite of the metabolism of diosmetin (10 μM) from CYP1A2. A 20 min CYP1A2 incubate was spiked with 1μM of 6OH luteolin. The difference in retention times, compared to the other traces, is due to a different column employed for this experiment.

Parameter	Enzyme		
	CYP1B1	CYP1A1	CYP1A2
Km (μM)	0.17	1.9	1.3
Kcat (pmol/min/pmol of enzyme)	2.8	23	10.7

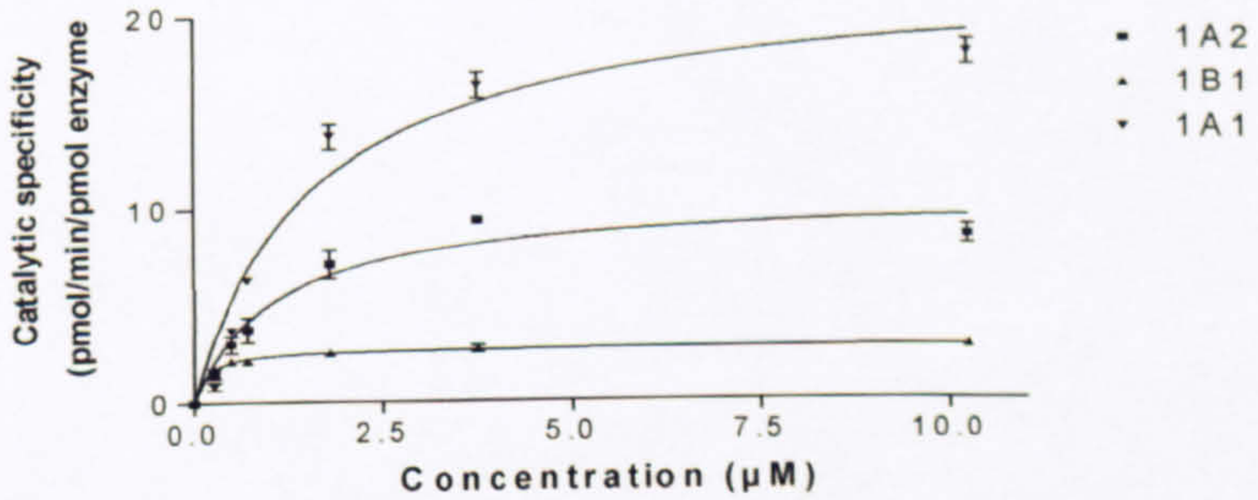
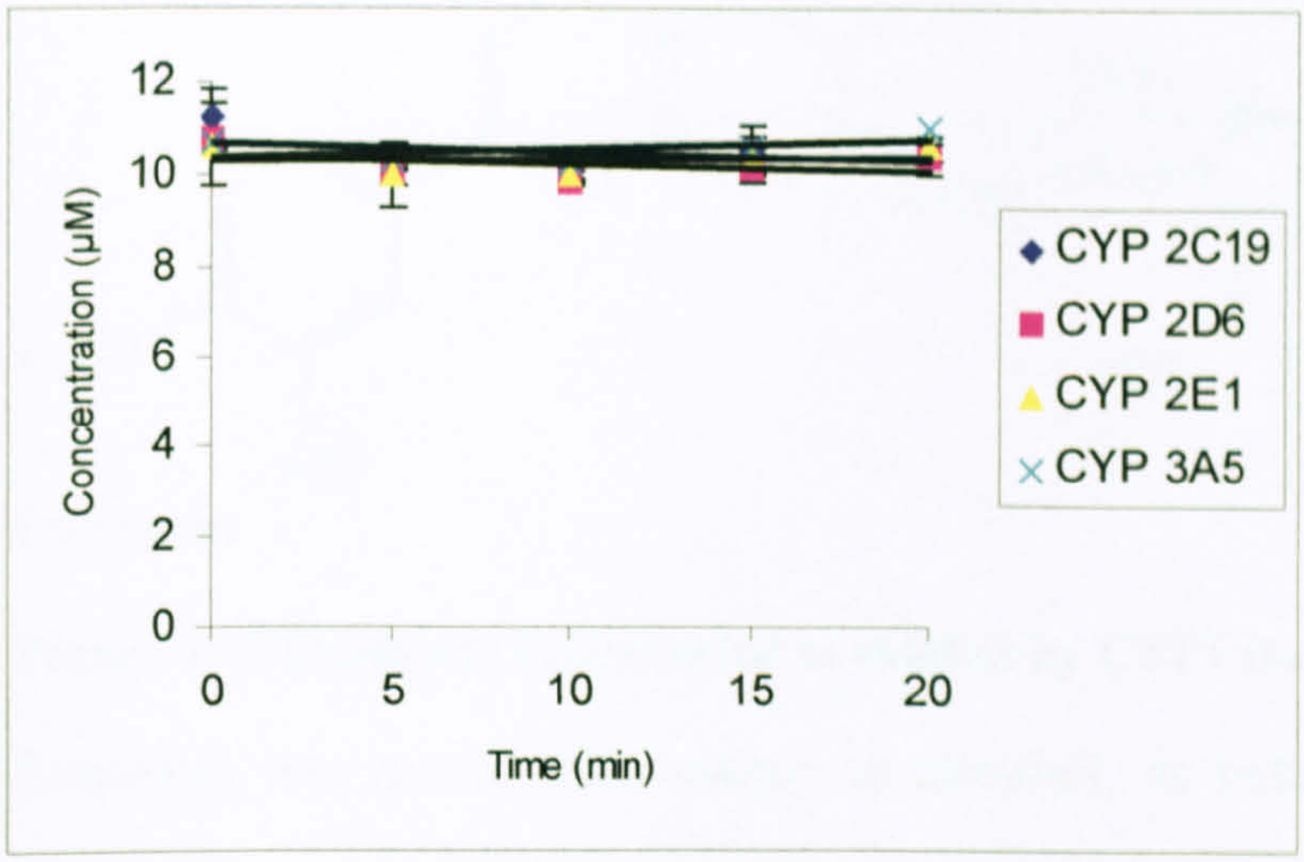


Figure 4.13 Michaelis Menten kinetics of the demethylation of diosmetin to luteolin. Error bars represent mean ± SD for n=3 determinations.



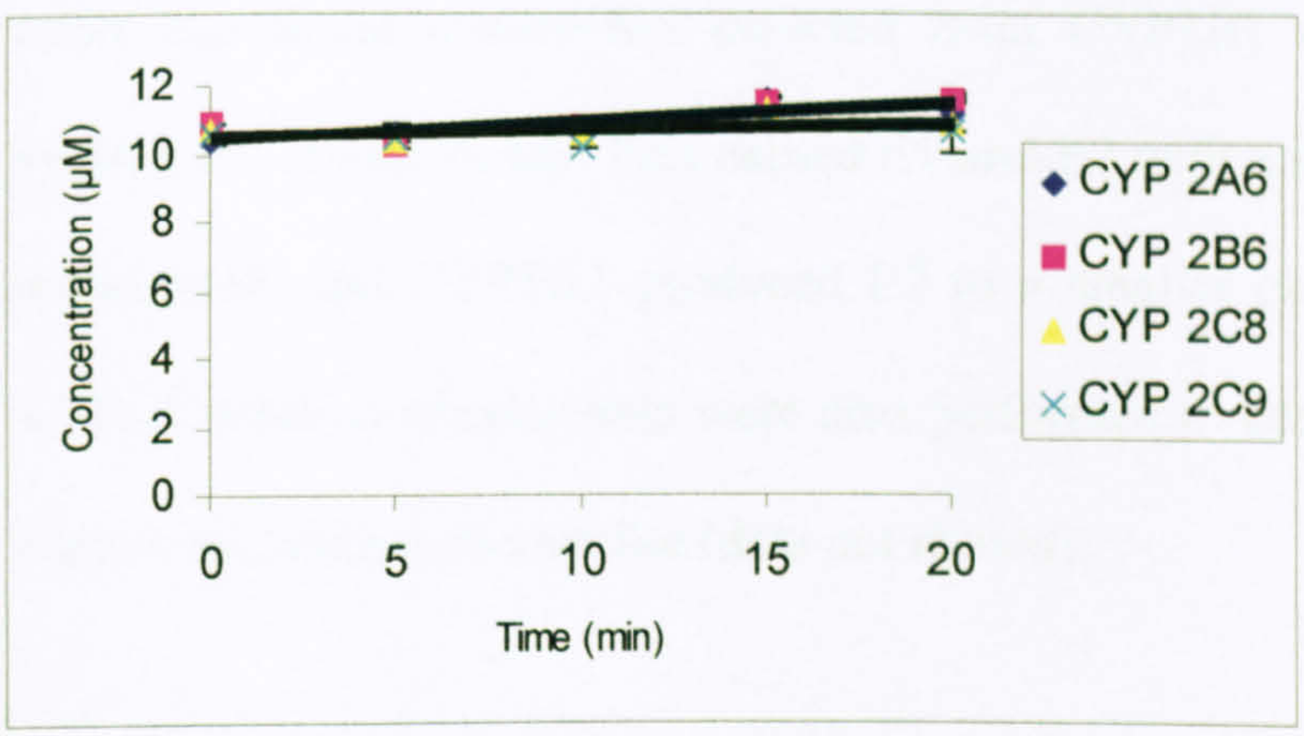
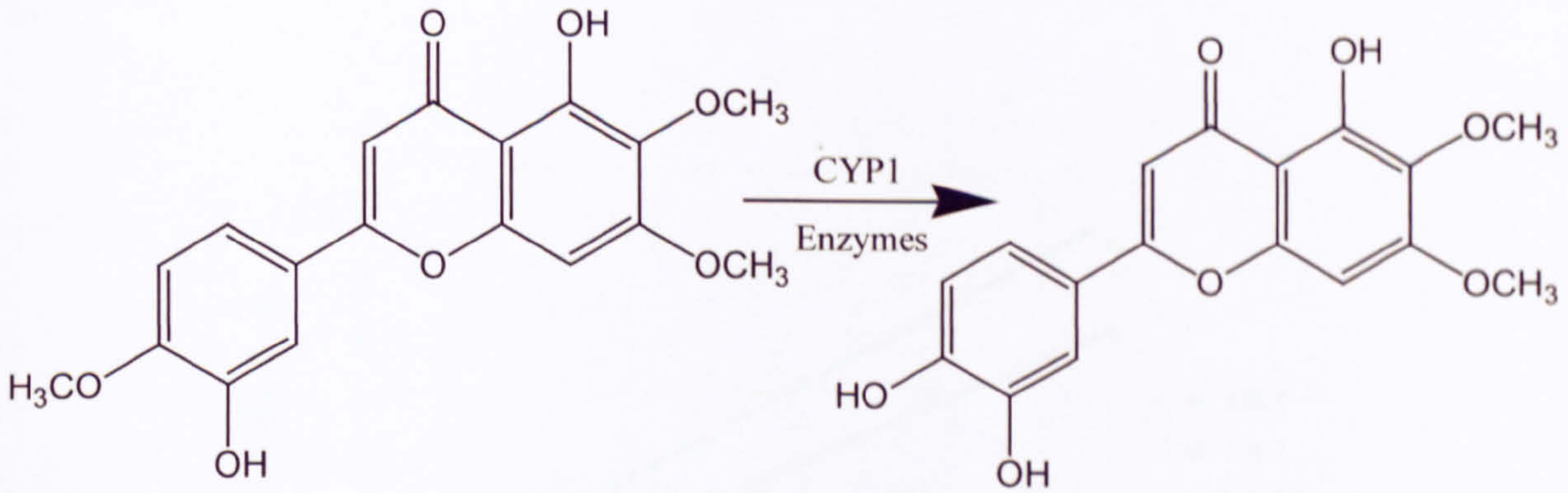


Figure 4.14 Rate of metabolism of diosmetin from a CYP panel. Error bars represent the min and max variation for n=2 determinations.

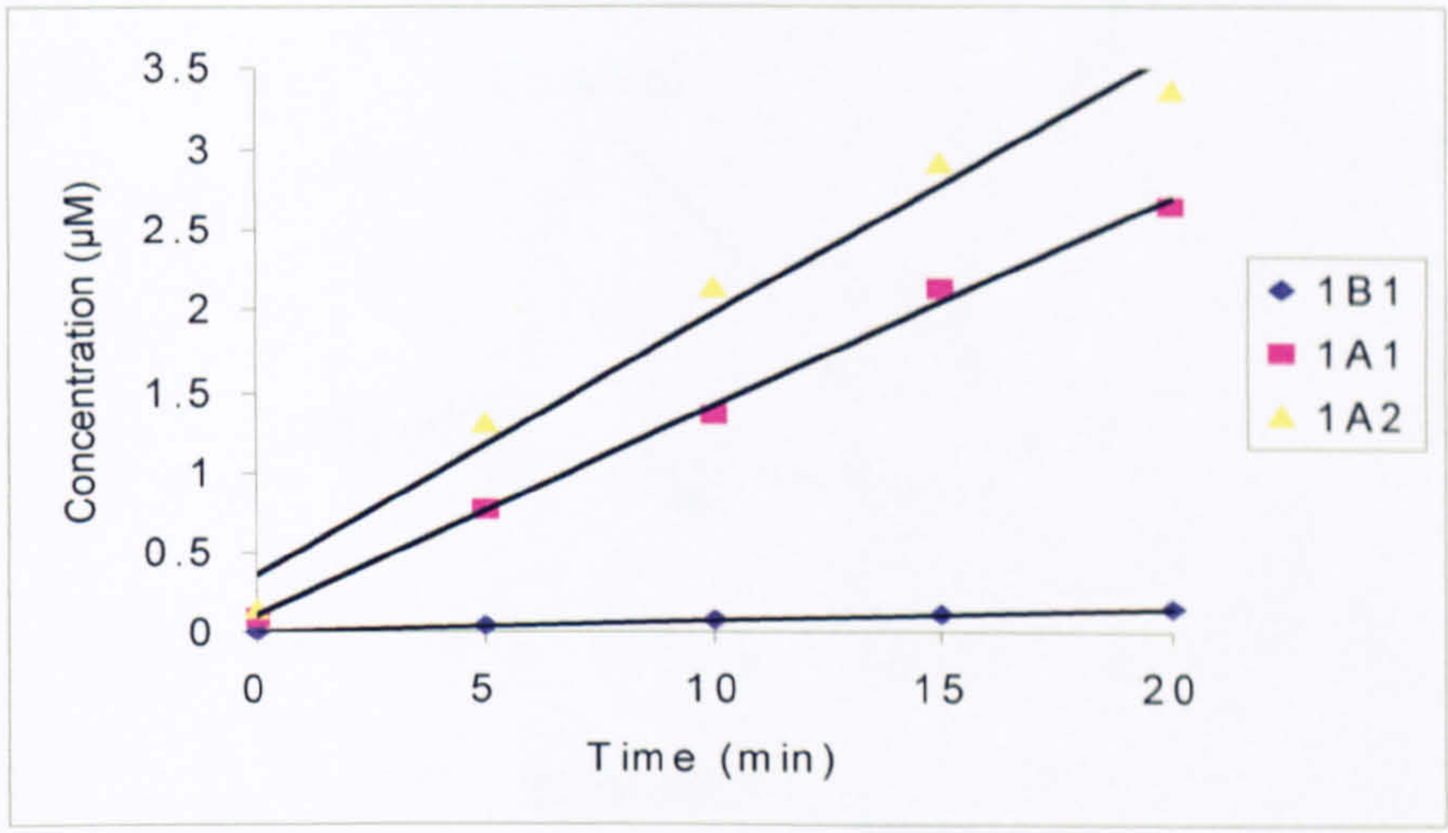


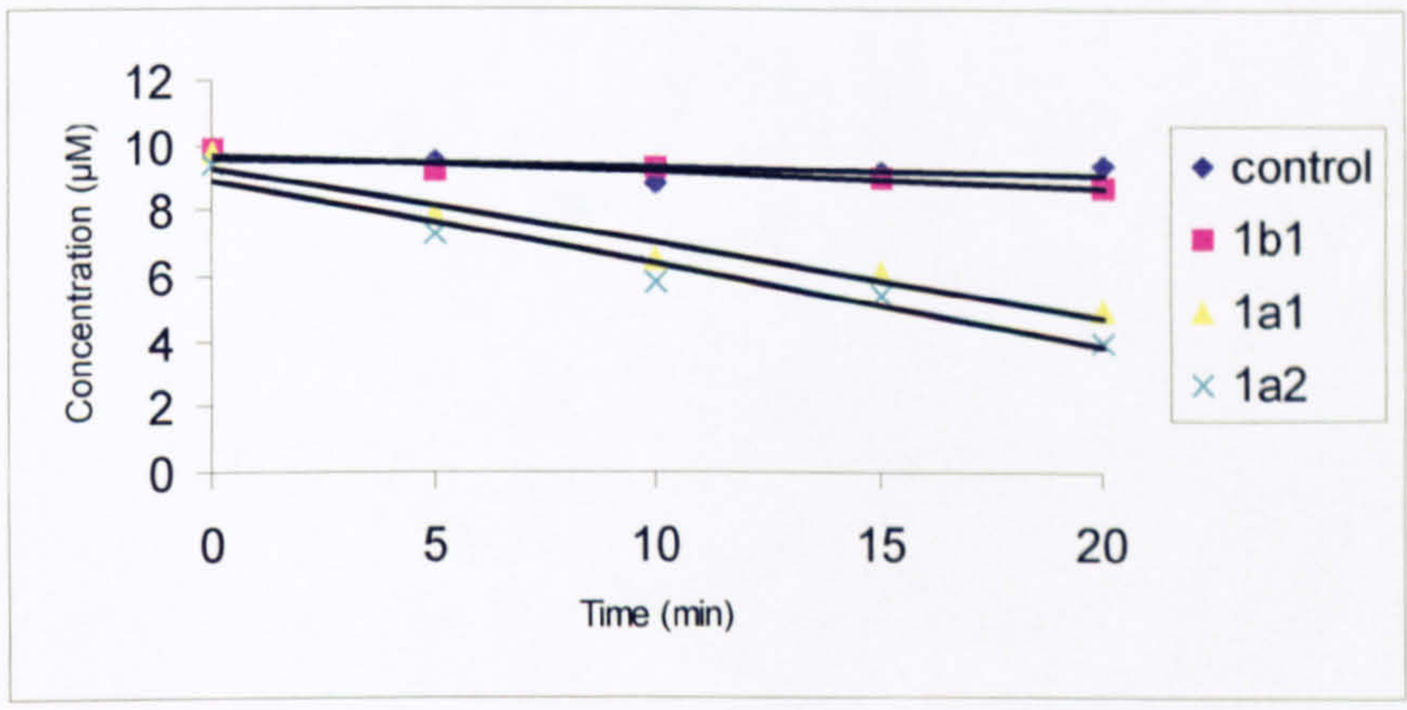
Eupatorin Cirisiliol

Figure 4.15 Eupatorin is converted to cirsiliol by CYP1 family enzymes

Eupatorin was metabolised mainly to cirisiliol, as indicated by the retention time, by CYP1A1 and CYP1A2 and to a smaller extent by CYP1B1 (Figure 4.16). There were no other significant metabolites detected from CYP1B1 metabolism, however CYP1A2 yielded two minor metabolites named E1 and E2 with a retention time of 7.5 and 7.7 min respectively and CYP1A1 produced E2 to a smaller extent and E1 to a larger (Figure 4.17). Coelution experiments were also performed to confirm the identity of cirisiliol as eupatorins primary metabolite (data not shown).

a)





b)

Figure 4.16 a)Rate of formation of cirisiliol by CYP1 family enzymes b) Rate of metabolism of eupatorin. Experiments were done in duplicate, a representative graph is shown.

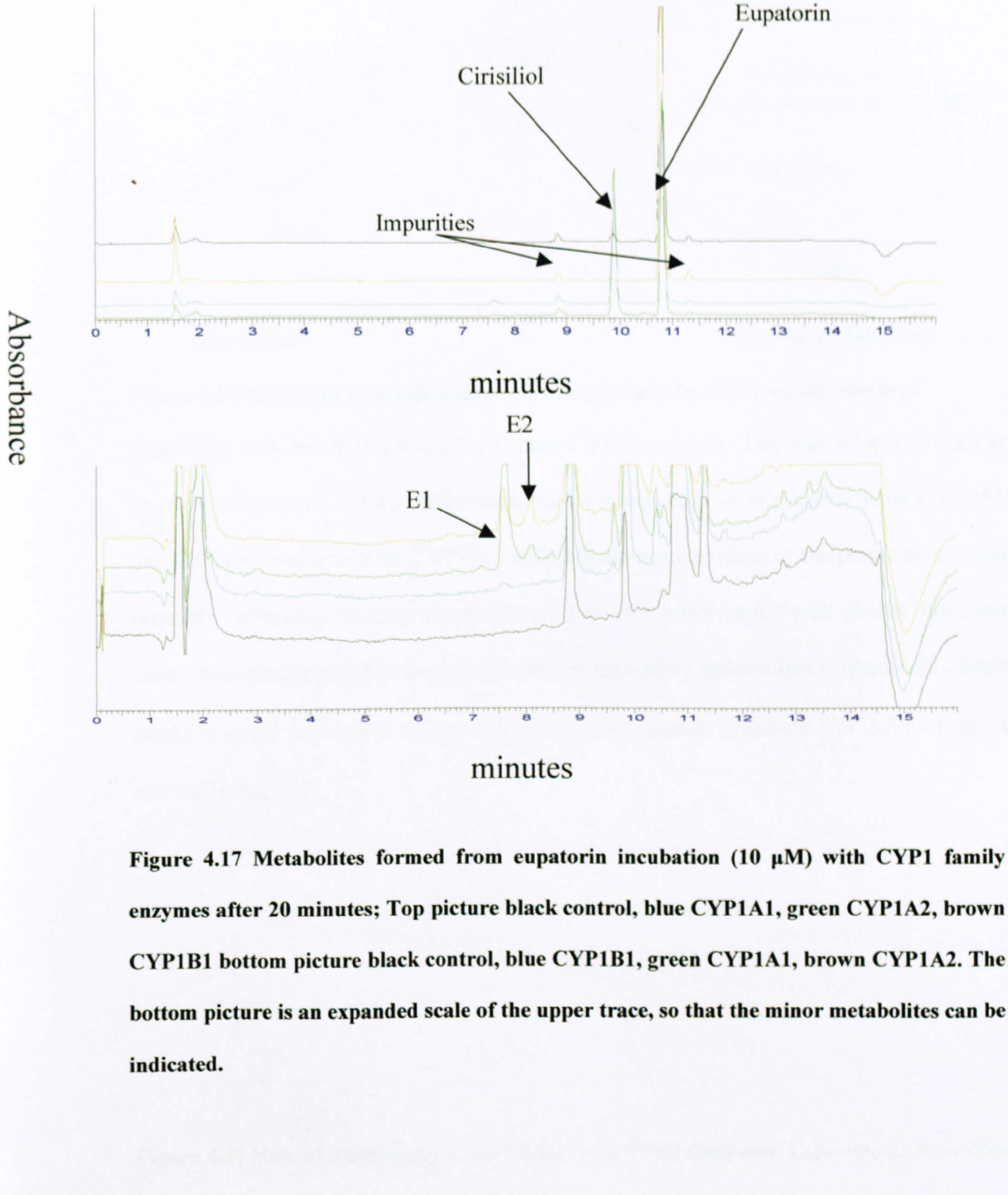
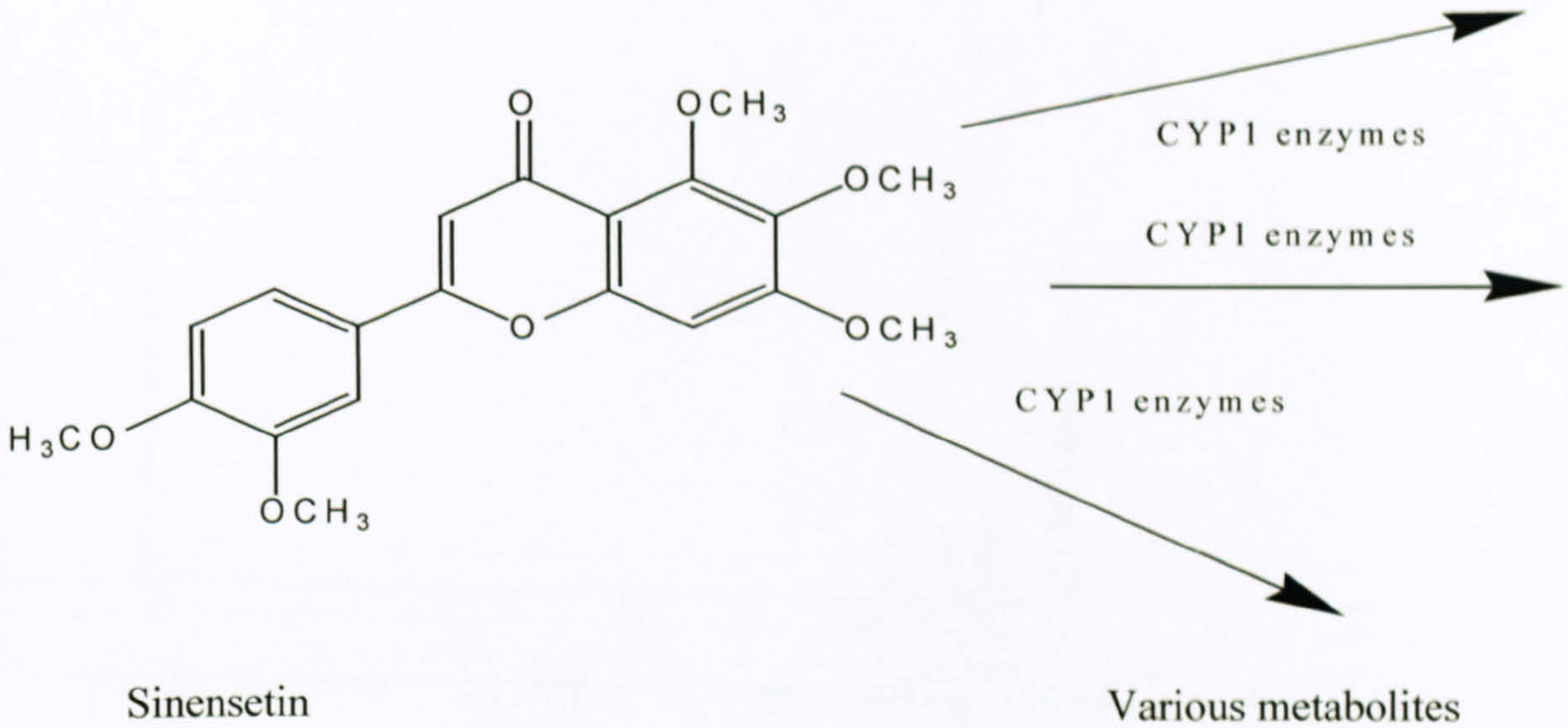


Figure 4.17 Metabolites formed from eupatorin incubation (10 μM) with CYP1 family enzymes after 20 minutes; Top picture black control, blue CYP1A1, green CYP1A2, brown CYP1B1 bottom picture black control, blue CYP1B1, green CYP1A1, brown CYP1A2. The bottom picture is an expanded scale of the upper trace, so that the minor metabolites can be indicated.



Sinensetin Various metabolites

Figure 4.18 Sinensetin is metabolised to various products by CYP1 family enzymes

Sinensetin was metabolised from all three CYP1 enzymes. The rate of metabolism is shown in Figure 4.19. Two unidentified major metabolites were detected from CYP1A1 and CYP1A2 and one from CYP1B1, with retention times close to the parent compound probably indicating primary metabolism, whereas smaller peaks with shorter retention times were also detected as a result of possible secondary metabolism (Figure 4.20). Such products could occur as a result of demethylation steps at positions 3′,4′,5,6,7 of the A and the B ring.

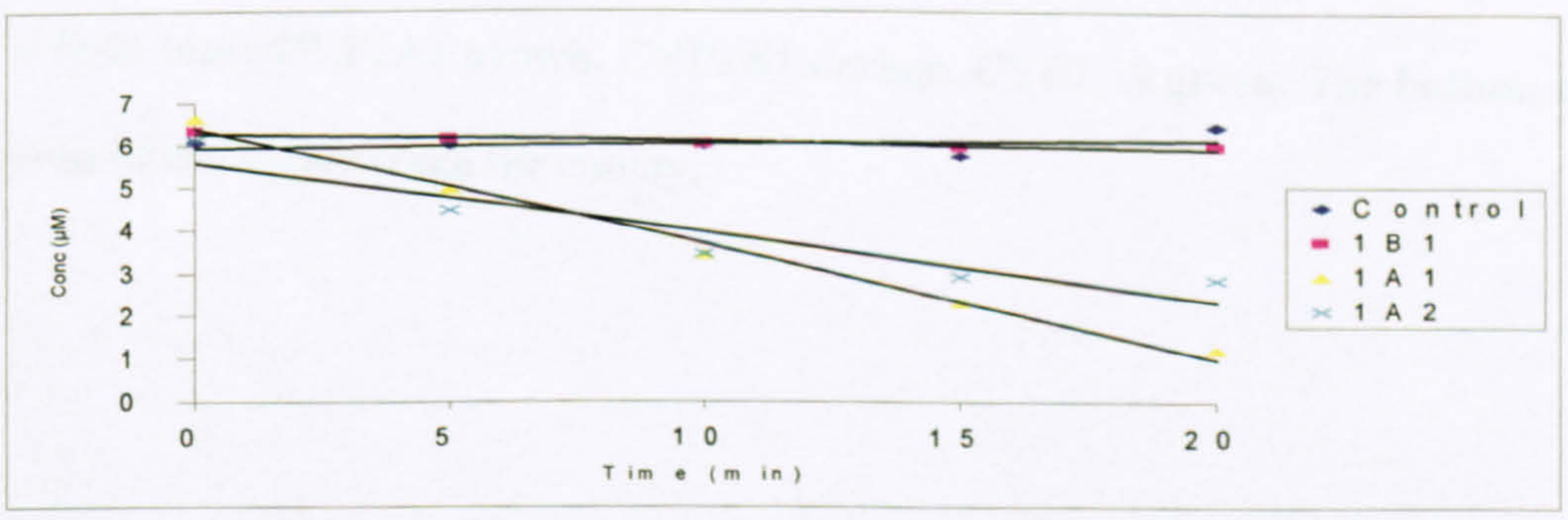


Figure 4.19 Rate of metabolism of sinensetin from CYP1 isozymes. Experiments were done in duplicate, a representative graph is shown

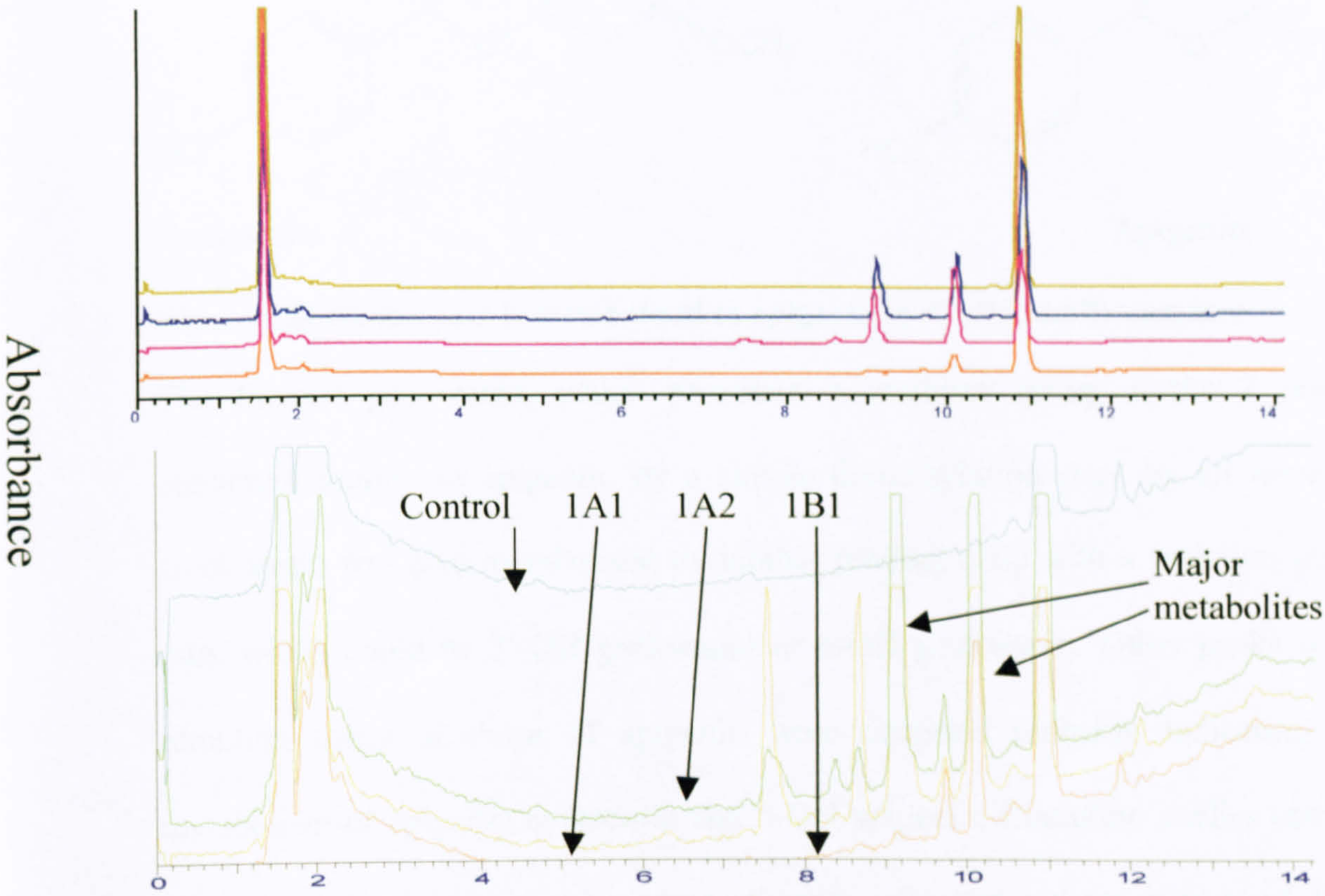
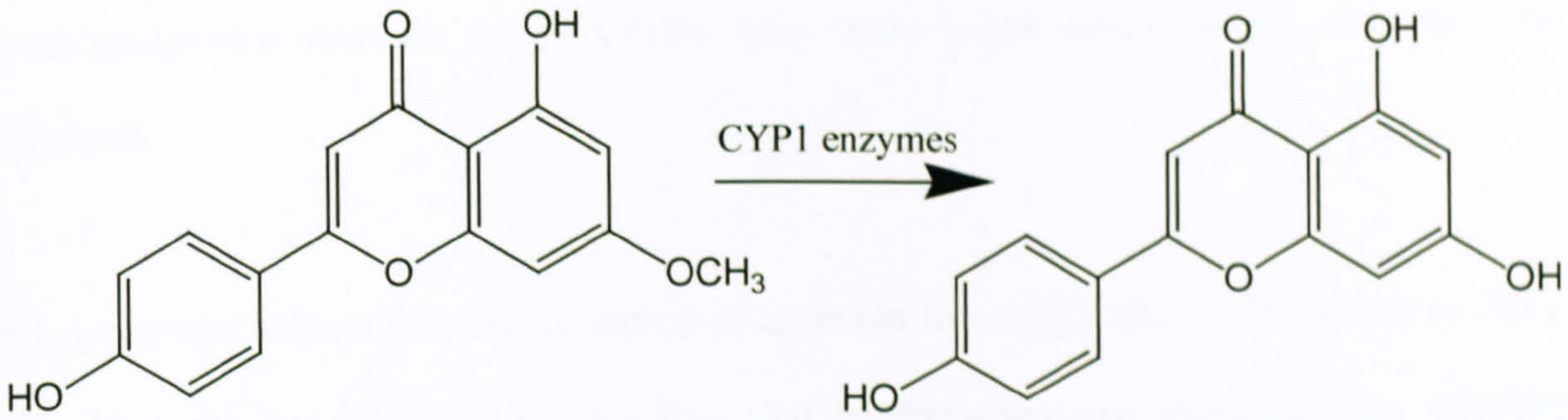


Figure 4.20 Metabolites formed from 20 min incubation of sinensetin (10 µM) with CYP1 isozymes: top : control brown, CYP1B1 orange, CYP1A1 blue, CYP1A2 pink. Bottom: control light blue, CYP1A1 brown, CYP1B1 orange, CYP1A2 green. The bottom trace is an expansion of the upper trace for clarity.



Genkwanin Apigenin

Figure 4.21 Genkwanin is metabolised to apigenin by CYP1 family enzymes

The flavone genkwanin, which possesses a methoxy group at the 7 position was converted mainly to apigenin, by a simple demethylation step, by all three enzymes. Genkwanin was also metabolised to another product (G1) with a retention time of 11.3 min, which could be 3′-OH genkwanin or 6-OH genkwanin. Other peaks with shorter retention times of those of apigenin were obtained probably indicating secondary metabolism of apigenin to luteolin and 6-OH apigenin. Coelution studies confirmed the identity of apigenin as main product of genkwanin metabolism from the CYP1 family enzymes (Figure 4.22).

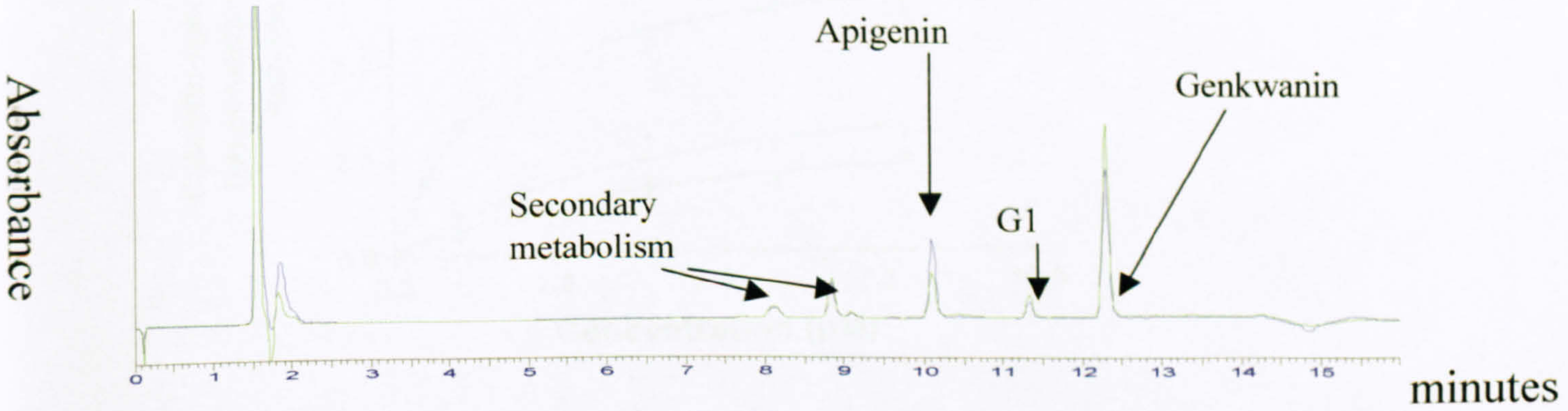


Figure 4.22 Coelution studies for the identification of apigenin as the primary metabolite of genkwanin (10 μM) from 20 min incubation with CYP1B1. The incubate was spiked with apigenin standard (1 μM). Same coleution was observed for the other two enzymes. Green

trace genkwanin incubate with CYP1B1, blue trace genkwanin incubate with apigenin standard.

It is important to note that the formation of apigenin from CYP1B1 mediated metabolism of genkwanin was greater over time than that for the other two enzymes. The opposite was observed for G1 product (data not shown). Thus one can speculate that apigenin is being preferentially formed by CYP1B1. The overall rate of metabolism of genkwanin was similar for all enzymes, probably due to secondary metabolism and formation of G1. Initial kinetic experiments demonstrate that genkwanin is a selective substrate for CYP1B1 (Km = 1.5 μM, Kcat = 7.8 pmol/min/pmol of enzyme, as opposed to Km = 4 μM, Kcat = 4.8 pmol/min/pmol of enzyme for CYP1A1 and 4.3μM and 3.5 pmo/min/pmol of enzyme for CYP1A2) (Figure 4.23).

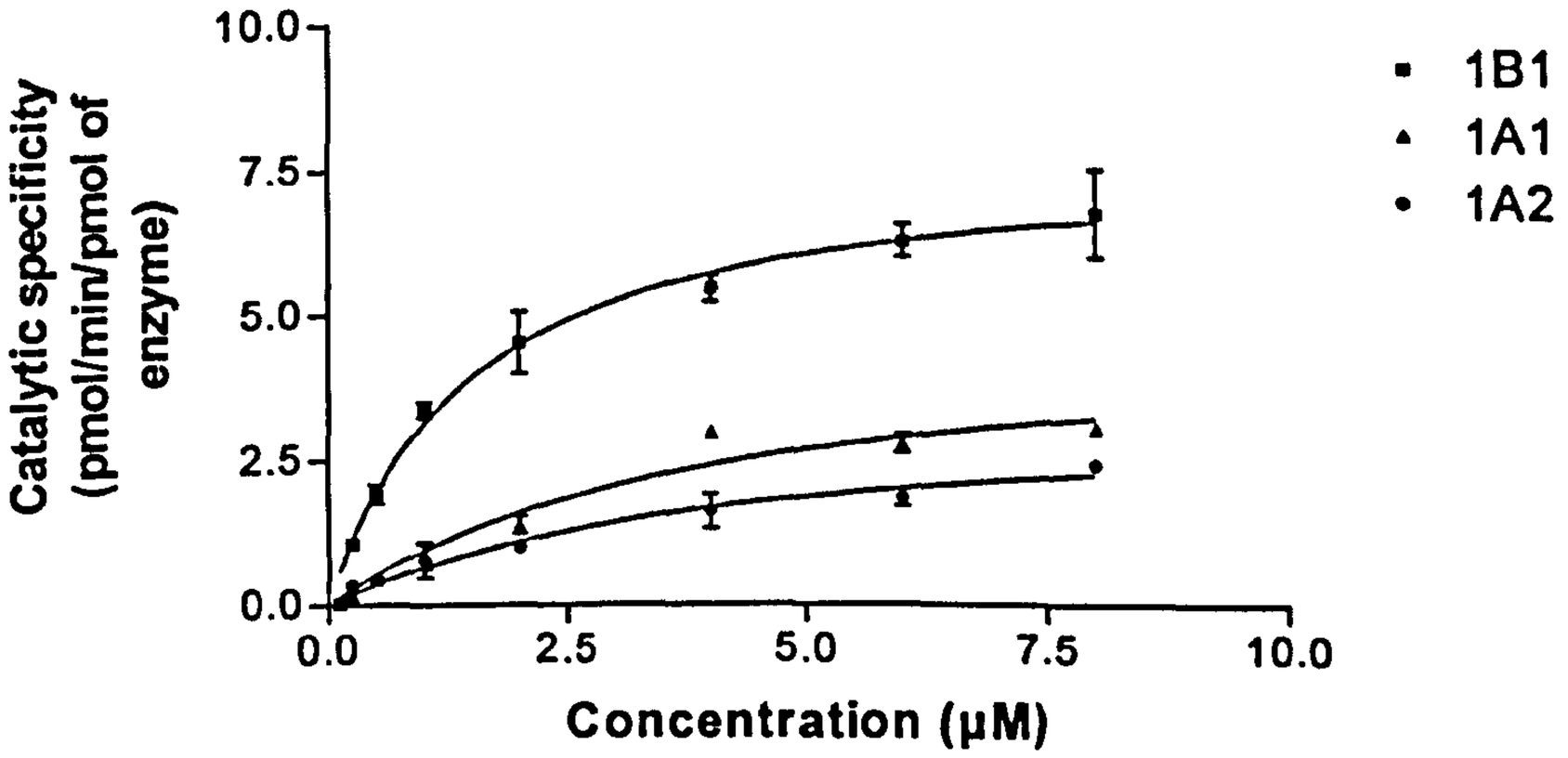


Figure 4.23 Enzyme kinetics of genkwanin CYP1 family catalysed demethylation. Error bars represent min and max variation for n=2 determinations.

In the present study evidence is provided for a possible pathway of CYP1 catalysed flavonoid biotransformation (Figure 4.24). The primary site of flavonoid metabolism of the flavones examined, was found to be the 3′ and 4′ position of the B ring and the 6 position of the A ring.

Baicalein was the only flavone which was not oxidised by the CYP1 family, whereas chrysin was metabolised to baicalein, genkwanin to apigenin, apigenin to luteolin and scutellarein (as a result of secondary metabolism). Comparison of the structures of these six compounds, suggests that the presence of substituents at positions 5 and 7 of the A ring is necessary for hydroxylation at the 6 position to occur. Moreover oxidation on the B ring requires pre-existing substitution as well (such as 4′ hydroxy in the case of apigenin) and as a result chrysin and baicalein which contain unsubstituted B rings were not oxidised from CYP1 enzymes to that position. Scutellarein was also metabolised exclusively to 6-hydroxyluteolin, which is in agreement with the above finding. Eupatorin and diosmetin possess methoxy substituents at the 6,7,4′ and 4′ postions respectively. The main route for these flavones metabolism was 4′ demethylation. Sinensetin was metabolised by CYP1 family enzymes but most of the metabolites could not be identified, due to the unavailability of authentic standards, although it seemed that the rate of metabolism increased as the number of methoxy substituents increased. Unidentified metabolites were also noticed in the metabolism of diosmetin and genkwanin (one in each study). However one can deduce that they would probably result from hydroxylation on the 6 position of the A ring, due to polarity differences with the other metabolites and the parent compound. The data presented in this chapter indicate that diosmetin and genkwanin are selective substrates of human CYP1A1 and CYP1B1

undergoing *O*-demethylations to the corresponding 4′-hydroxylated and 7-hydroxylated flavone respectively.

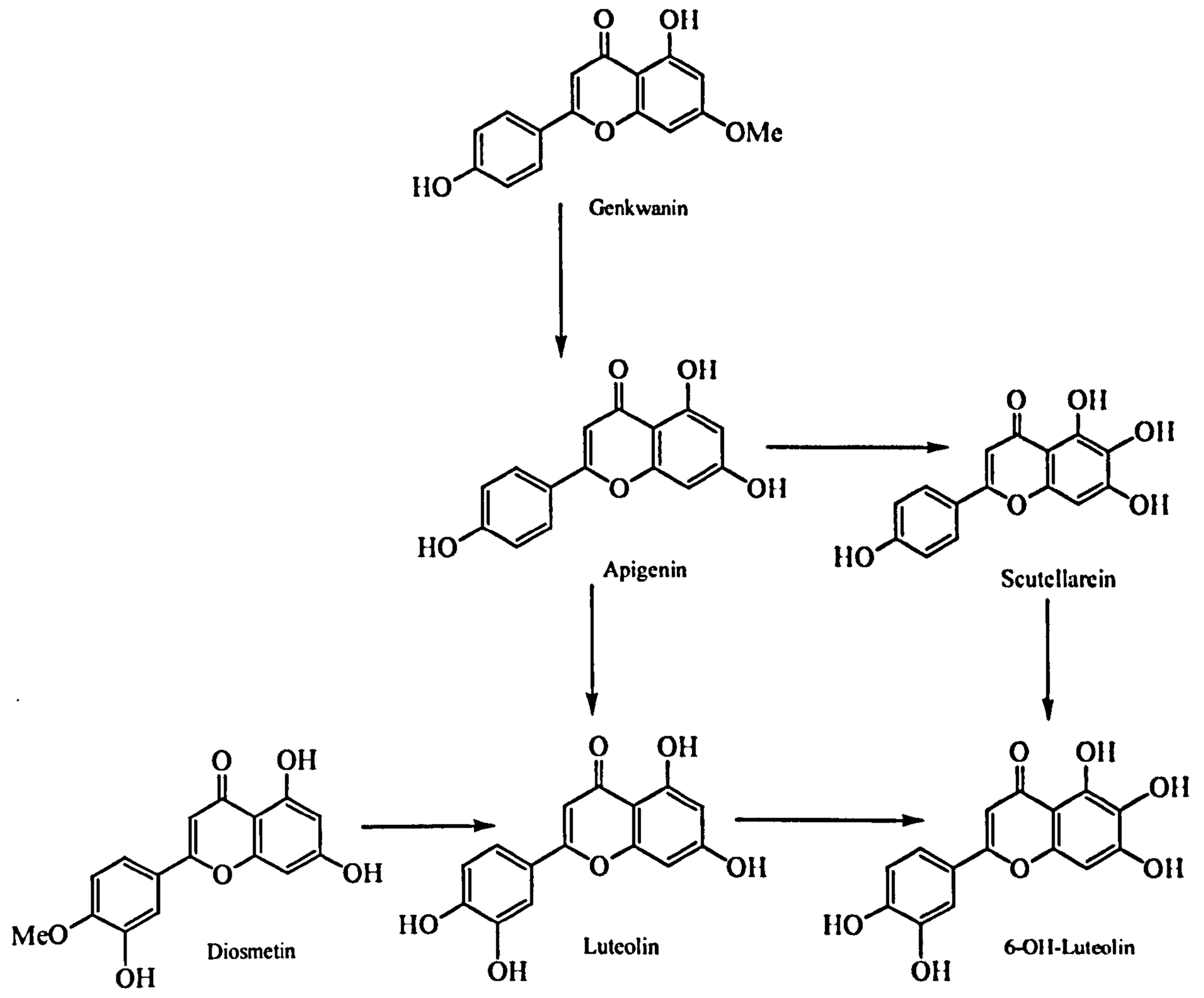


Figure 4.24 Possible bioactivation pathways of dietary flavones by CYP1 isozymes

There are a few studies in the literature regarding the metabolism of flavonoids from CYP1 enzymes and the evidence is sometimes contradictionary. For example it was shown from Otake and Walle (2002) that chrysin was not oxidised by pooled human liver microsomes and recombinant CYP1A1 and CYP1A2 enzymes. Galijatovic et al. (1999) showed also that chrysin was not oxidised by CYP1 enzymes, but instead it was conjugated to form glucuronides and sulphated metabolites in HepG2 and CaCo-2 cell

lines. The same metabolic profile was observed for apigenin. The authors suggested that conjugation reactions were more important in the non induced HepG2 and CaCo-2 cell lines. In another study it was shown that chrysin is metabolised to a large extent to apigenin by uninduced and induced Aroclor-1254 rat liver microsomes and to luteolin to a minor extent (Nielsen et al., 1998). Here it is shown that chrysin is metabolised to baicalein and bacalein is not metabolised by recombinant human CYP1 family enzymes. CYP1A1 and CYP1A2 have been shown to catalyse to a small extent the hydroxylation of 17β-oestradiol to the C-15a and 16-a position respectively (Badawi et al., 2001). Since 17β-oestradiol can be overlayed on chrysin that would explain why chrysin was metabolised to the 6 position of A ring.

Scutellarein was metabolised to 6-hydroxyluteolin. Resveratrol was shown to be metabolised to piceattanol and to two other metabolites (Potter et al., 2002) resulting from hydroxylation on the second aromatic ring. In the case of scutellarein the 6 position of the A ring is already hydroxylated, hence the only position left vacant is the 3′ position of the B ring. These results are in agreement with Potter et al. (2002). Moreover Wilsher (2003) showed that luteolin is exclusively metabolised to 6 hydroxy luteolin by CYP1B1. No other metabolite was detected in the latter study. Hydroxylation of the flavones might occur in positions 2′, 5′ and 6′ of the B ring, however this type of reaction has never been reported to date by CYP1 family enzymes, in this subclass of natural products.

Diosmetin was converted to luteolin and to two other metabolites. One of the other metabolites was shown to be 6-hydroxyluteolin (only produced by CYP1A1 and CYP1A2). Since Wilsher showed, that 6-hydroxyluteolin is produced from luteolin to a minor extent, exclusively from CYP1B1, the above findings suggest that diosmetin is

metabolised to luteolin and 6-hydroxydiosmetin. 6-hydroxydiosmetin is then further converted to 6-hydroxyluteolin. CYP1B1 could convert the luteolin produced from diosmetin 4′ demethylation to 6-hydroxyluteolin. However the amount of luteolin formed was probably too low for its 6 hydroxylated metabolite to be produced in detectable concentrations from this assay. Breinholt et al. (2002), showed amongst other flavonoid compounds that apigenin is converted to luteolin, hersperitin (the flavanone equivalent of diosmetin) to eriodictyol (the flavanone equivalent of luteolin) and tamarixetin (the flavonol equivalent of diosmetin) to quercetin (the flavonol equivalent of luteolin) by human liver microsomes and recombinant human CYP1A2 enzymes expressed in *E.coli.* The results presented here are in agreement with this study.

There are few studies (Nagao et al., 2002, Martini et al., 2004) on the biological activity of the remaining methoxylated flavones investigated in this chapter (eupatorin, genkwanin and sinensetin) and none of them has examined the the substrate specificity of these compounds for CYP1 family enzymes. It was shown that eupatorin follows the same demethylation pattern of diosmetin at least for one of the metabolites (cirsiliol) and that genkwanin is converted to apigenin through demethylation of the 7 position of the A ring. It could be speculated for sinensetin that it would undergo possible demethylation reactions in the 4′ position of the B ring and in the 6 and 7 positions of the A ring as its primary metabolic route. Secondary metabolites are also possible.

The flavonoids investigated have already been implicated as chemopreventive agents and several possible explanations for such properties have been proposed. In this chapter it has been demonstrated that this is likely due to CYP1 enzyme catalysed bioactivation. Apigenin produced both G_2/M and G_0/G_1 cell cycle arrest in asynchronous human diploid

fibroblasts, mediated by inhibition of Cdk2 kinase and phosphorylation of Rb protein (Lepley, 1997), and was shown to induce apoptosis in HL-60 (human promyelotic leukaemia) cells, through a rapid induction of caspase-3 activity (Wang et al., 1999). Moreover it inhibited eukaryotic topoisomerase I catalysed DNA religation by forming a ternary complex with Topo I and the DNA substrate during the cleavage reaction, (Boege et al., 1996). This evidence could explain the anticancer properties of genkwanin by conversion to apigenin via CYP1 enzymes. Similarly, luteolin increased p21 levels in human LNCap prostate cancer cells, and caused G_1 cell cycle arrest in human melanoma cells OCM-1 (Wang et al., 1999). It has also been shown to inhibit the catalytic activity of eukaryotic DNA topoisomerase I interacting with both free enzyme and substrate DNA, and be a very potent inhibitor of protein kinase C and PI-3 kinase (Chowdhury et al., 2002, Kobayashi et al., 2002). These findings can offer more insight on the chemopreventive activities of flavonoids, such as diosmetin and apigenin, which are metabolised to luteolin through CYP1 enzymes expressed in tumours. Finally luteolin itself can be converted to 6-hydroxyluteolin by the same enzymes, which has also been implicated in antiproliferation of murine melanoma (B16F10), uterine carcinoma (HeLa) and human castric adenocarcinoma (MK-1)(Nagao et al., 2002). Similar anticancer properties have been reported for baicalein, which can explain the activity of chrysin (Liu et al., 2002).

In conclusion the results reported in this chapter provide the first supportive evidence for the hypothesis that CYP1 family enzymes act as tumour suppressor enzymes, via metabolism of dietary flavones to different products. The anticancer properties of the resulting metabolites require more extensive studies.

5. BIOACTIVATION OF NATURAL FLAVONES IN MDA-MB 468 AND MCF7 CELLS

5.1 Introduction

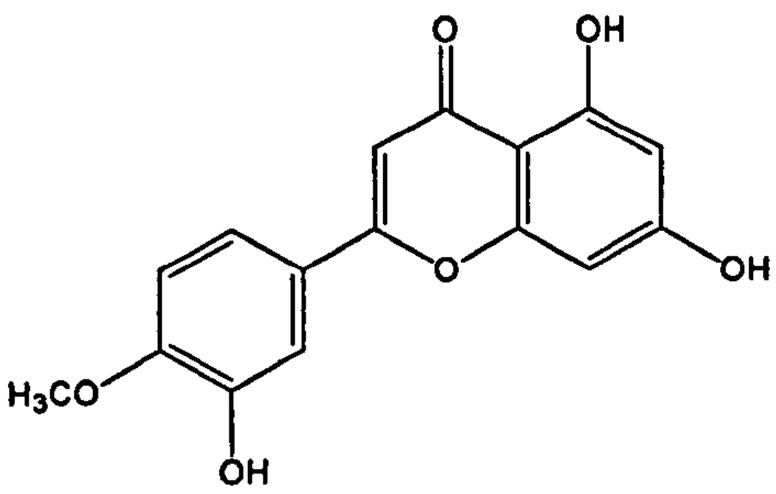
Amongst the different mechanisms by which flavonoids can exert their anticarcinogenic effects, is their ability to supress tumour growth. The antiproliferative/cytotoxic activity of various classes of flavonoids against cancer cells has been an extensive area of research for the last 15 years. It has provided structure activity and other mechanistic information regarding the way by which flavonoids act as chemopreventative agents. Flavonoids such as quercetin and baicalein have been shown to have growth inhibitory activity in a variety of tumour derived cell lines (Kawaii et al., 1999).

In the previous chapter it was shown, by enzymatic assays, that most of the flavones examined were metabolised to different products by CYP1 family isozymes. In order for a proof of principle concept to be established, it is necessary to obtain the same result in cell based assays. In addition, we wanted to investigate, whether this type of biotransformation would enhance the antiproliferative action of the flavonoids, towards the cancer cells.

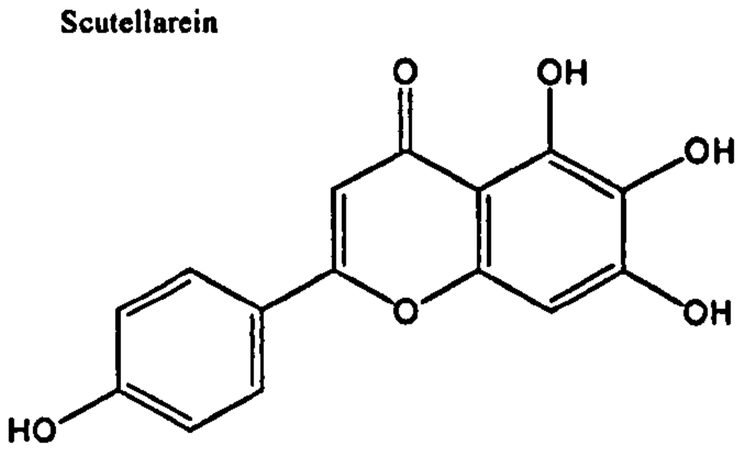
Previous work in our laboratory had concentrated on the selection of a cell line, which would express CYP1 family enzymes and thus could be used as a screening model to test bioactivation of natural products and related synthetic analogues. MDA-MB 468, which is a breast adenocarcinoma cell line (www.lgcpromochem-atcc.com), was chosen. Western immunoblotting was perfomed, in order to identify, whether the main CYP1 isoform expressed in MDA-MB 468 cells is CYP1B1. MCF10A cells, which were derived from primary breast cultures (www.lgcpromochem-atcc.com), were used as a negative control cell line, to identify possible toxicity of the flavonoids to normal cells.

The metabolism of the flavones was investigated in MDA-MB 468 and MCF10A cells using HPLC and their cytotoxic action was evaluated using the MTT assay.

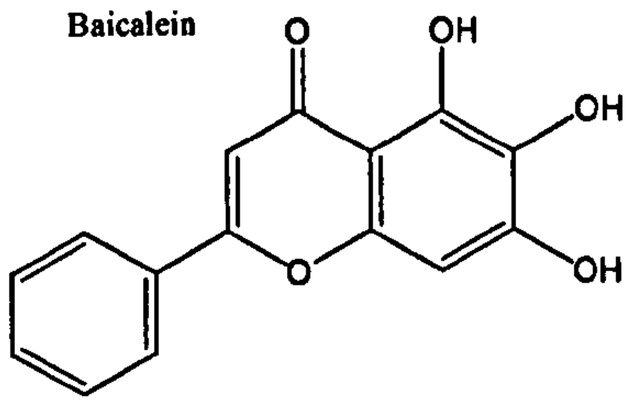
Diosmetin



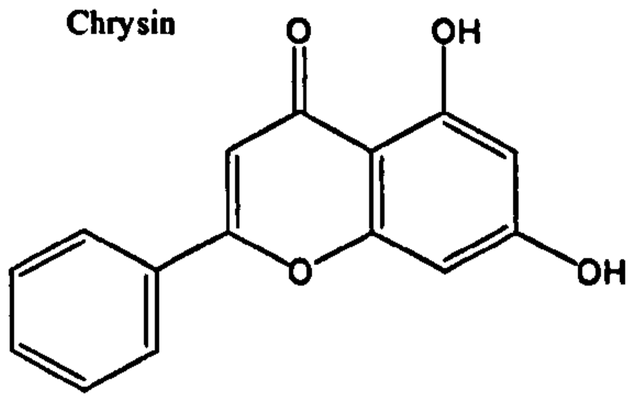
Scutellarein



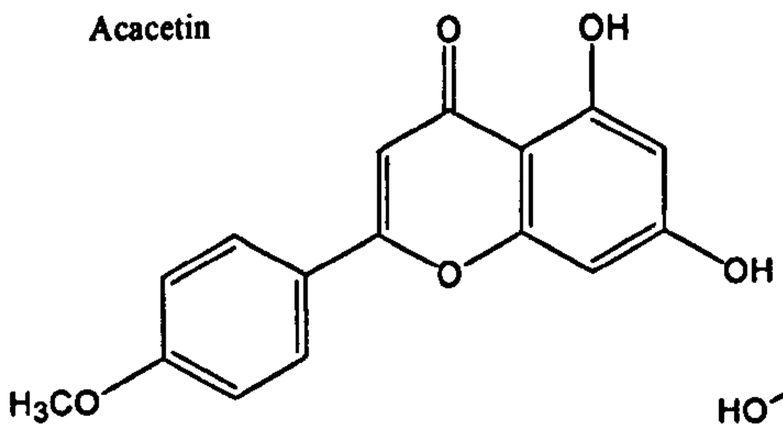
Baicalein



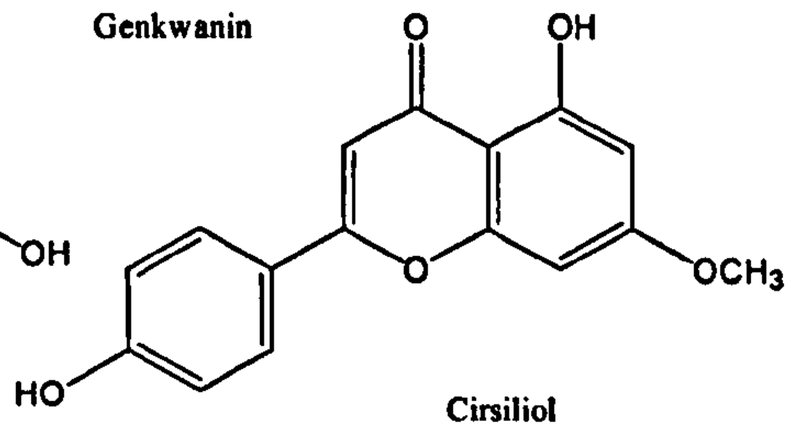
Chrysin



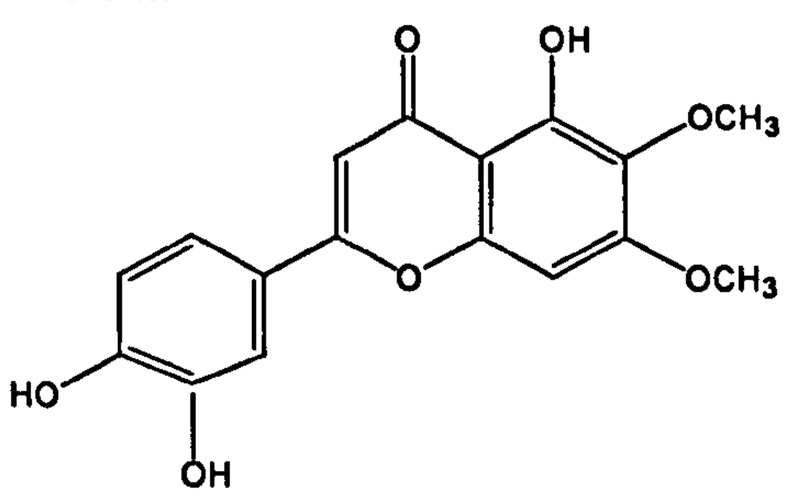
Acacetin



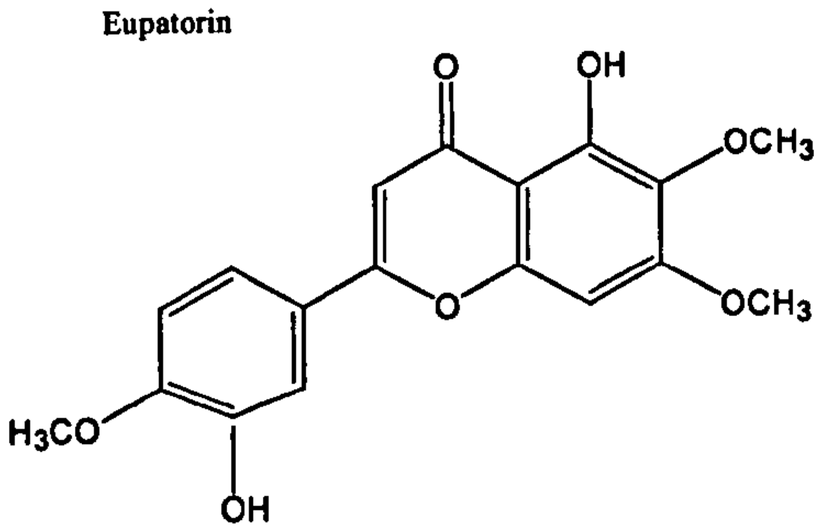
Genkwanin



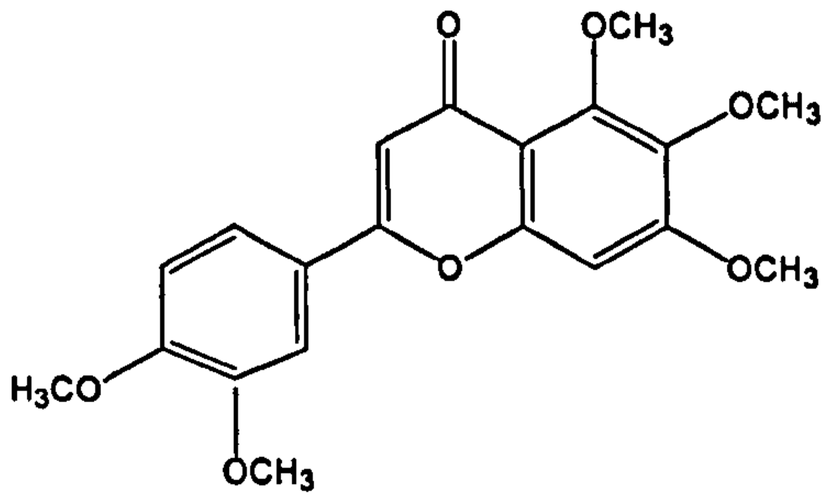
Cirsiliol



Eupatorin



Sinensetin



Eupatorin-5-methyl ether

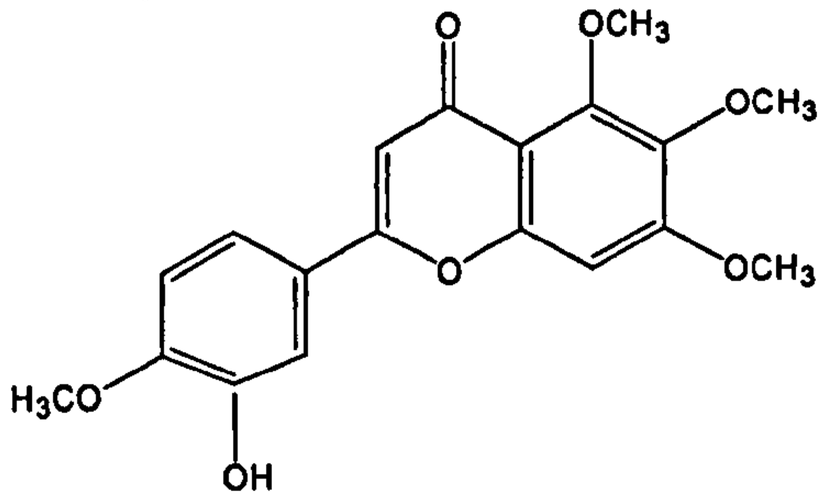
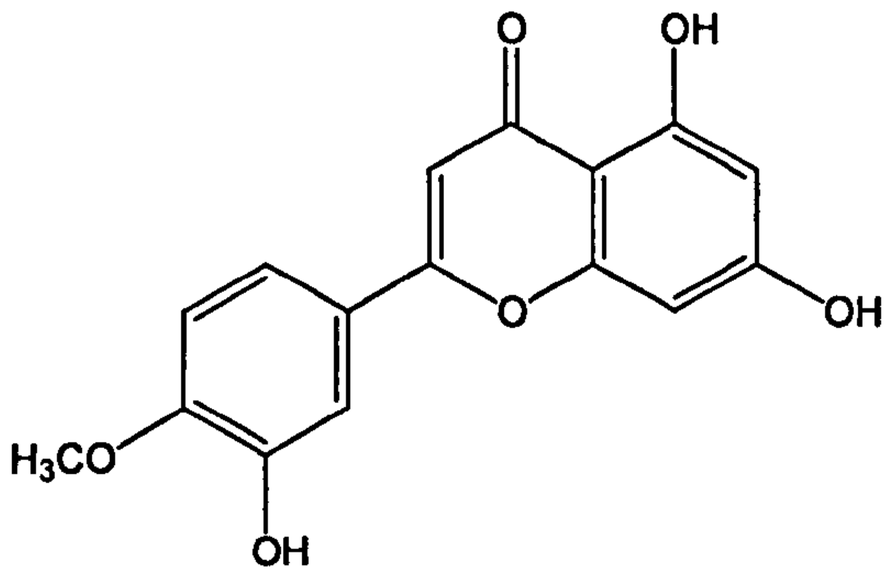
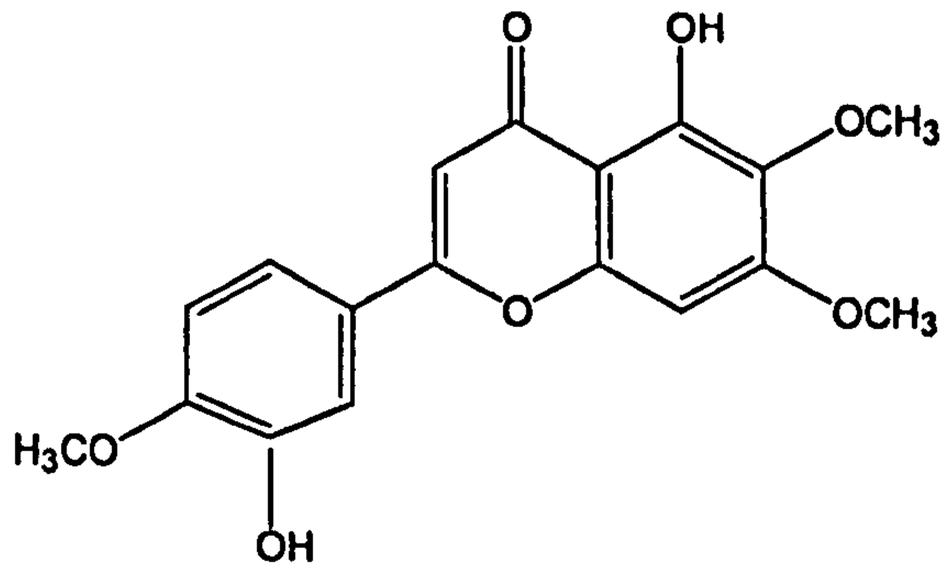


Figure 5.1 Structures of the compounds assayed by MTT

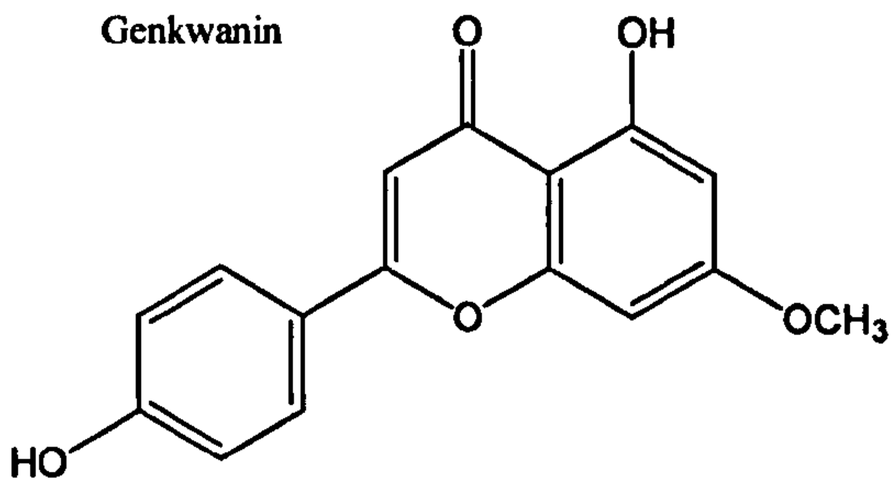
Diosmetin



Eupatorin



Genkwanin



5.2 Structures of the compounds analysed by HPLC

5.2 Results and discussion

MDA-MB 468 cells express CYP1 family enzymes as shown by EROD assay (Figure 5.3). Little or no EROD activity was observed in MCF10A cells. Therefore these two cell lines could be used to assess the metabolism of natural products, from CYP1 family enzymes. The antibody used in the western blotting did not detect any bands corresponding to molecular weights of 60 kDa, which would be expected for CYP1B1, in MDA-MB 468 cells. A band was obtained from the CYP1B1 microsome, used as a positive control, purchased from Gentest, at 60 kDa (Figure 5.4). A lot of background reactivity appeared from proteins present at lower molecular weights in the MDA-MB 468 and the CYP1B1 microsome samples (Figure 5.4).

HPLC analysis showed that genkwanin and diosmetin are metabolised in MDA-MB 468 and not in MCF10A cells. Diosmetin produced two minor metabolites in MDA-MB 468 cells (Figure 5.5). The one eluted at about 8.7 min and was luteolin as shown by previous data in chapter 4 and the other eluted at 7 min and was probably D2, i.e. 6 hydroxy diosmetin as discussed previously. Genkwanin was metabolised to three metabolites with retention times of 11, 8.4 and 7 min respectively (Figure 5.5). A peak was obtained at 10 min in both cell lines, which was an impurity present in the standard (data not shown). Thus we cannot make any definite conclusions as to whether; the last two metabolites are appearing from the metabolism of genkwanin or of the impurity. Eupatorin was also metabolised in MDA-MB 468 cells. One of the metabolites was cirsiliol, as seen from the relative retention time difference and from the enzyme assays discussed in chapter 4 (Figure 5.5). The other metabolite eluted at 7 min. No metabolism was observed in MCF10A cells. An impurity was present in the standard, eluting at 8.5 min.

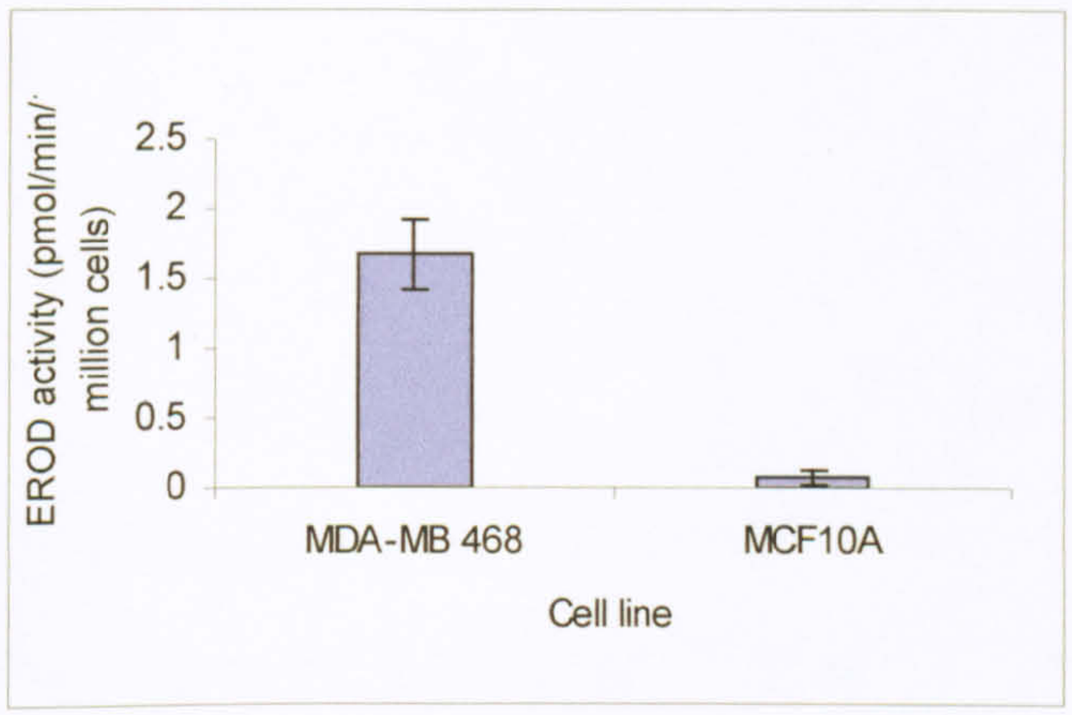


Figure 5.3 EROD activities of MDA-MB 468 and MCF10A cells. Blue bars represent mean ±standard deviation of three individual experiments.

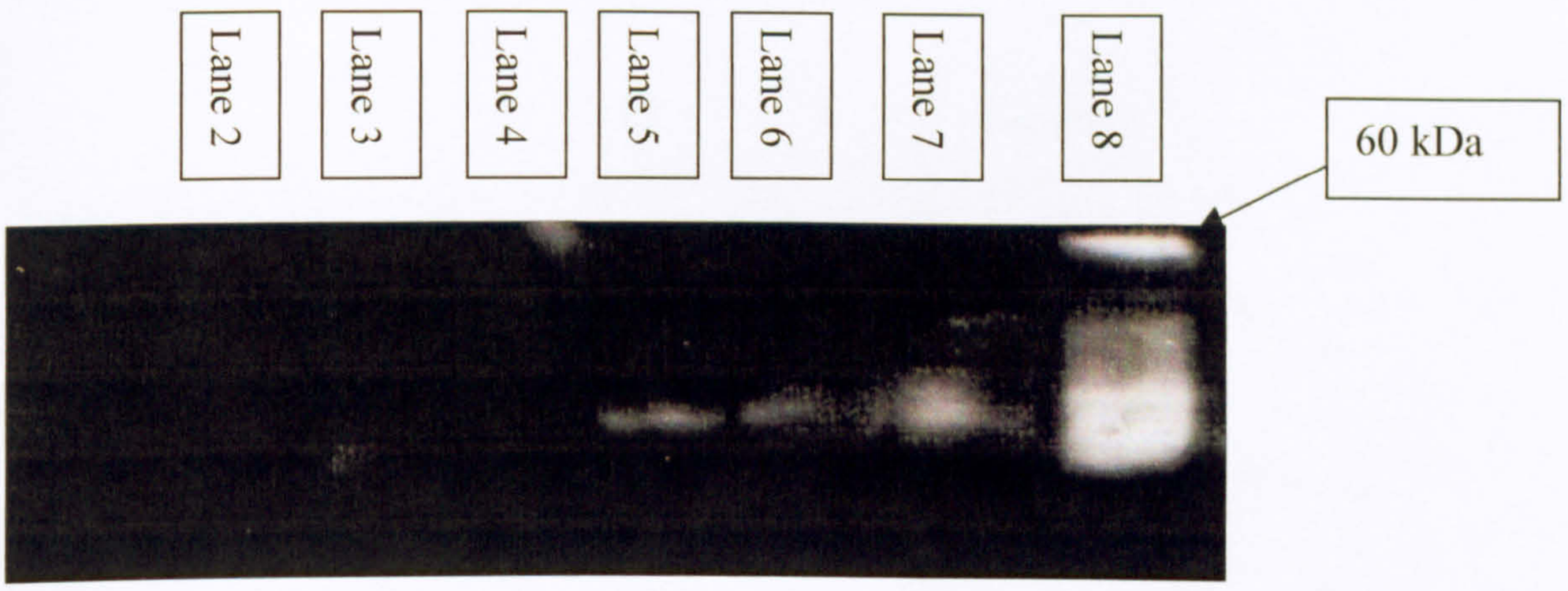


Figure 5.4 Protein immunoblotting of MDA-MB 468 and MCF10A cells. From left to right; lane 2-4; MCF10A protein extracts, lane 5-7; MDA-MB 468 protein extracts, lane 8; CYP1B1 microsome positive control from Gentest). The experiment was repeated at least three times.

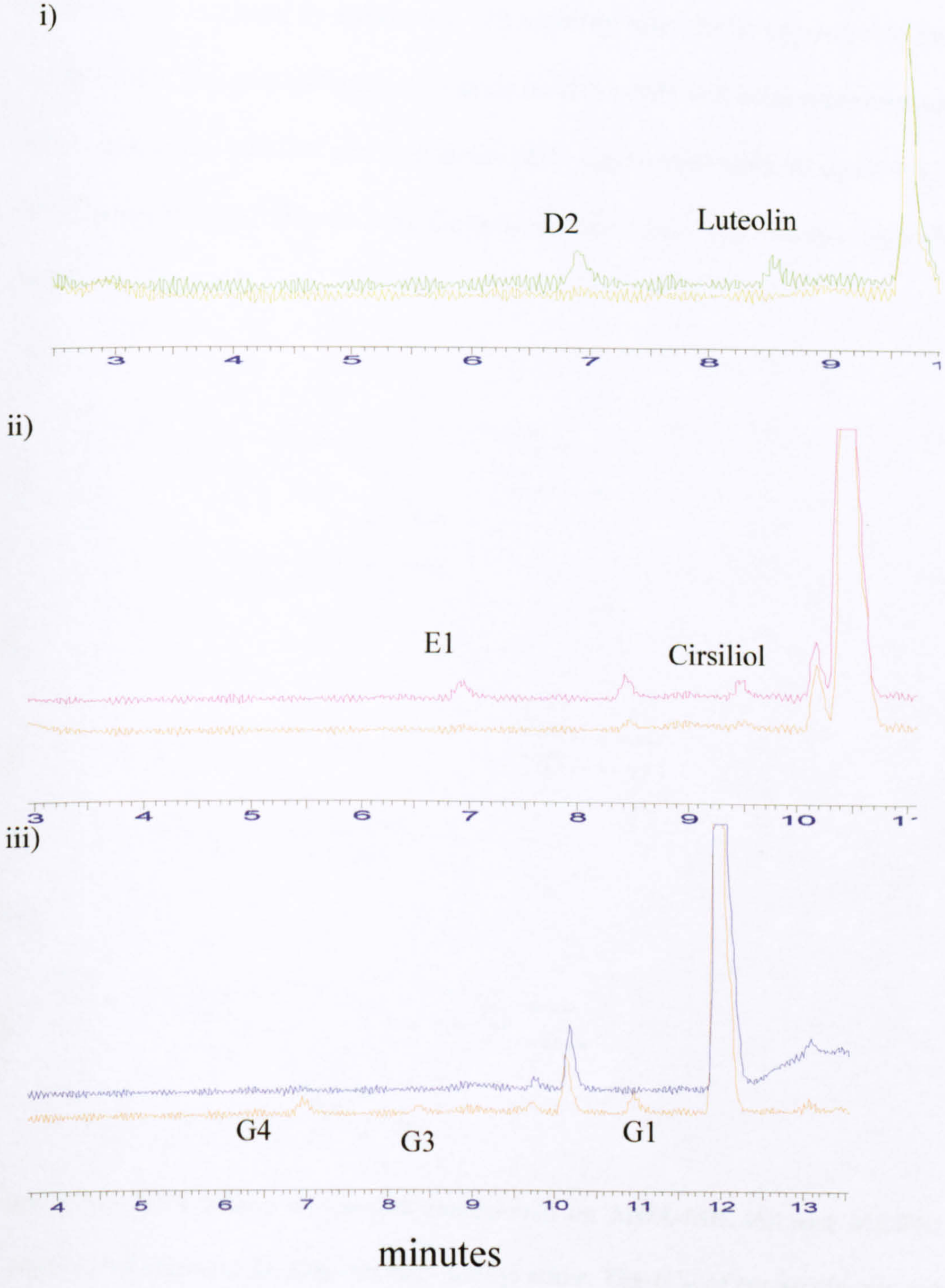


Figure 5.5 Metabolism of flavones (10 μM) in MDA-MB 468 and MCF10A cells. i) Diosmetin; green; MDA-MB 468 brown; MCF10A. ii) Eupatorin; red; MDA-MB 468 orange; MCF10A. iii) Genkwanin; blue; MCF10A 0range; MDA-MB 468. Experiments were done in duplicate.

The ability of the natural products to inhibit the growth of MDA-MB 468 and MCF10A cells was investigated by measuring cell viability after 96 hr exposure of the cells to the compounds. The most potent compounds in MDA-MB 468 cells were eupatorin-5 methyl ether, eupatorin, cirsiliol and sinensetin (data not shown) with IC_{50s} of 0.2, 0.4, 0.2 and 0.7 μM respectively (Figure 5.6). Genkwanin and diosmetin also inhibited the growth of breast

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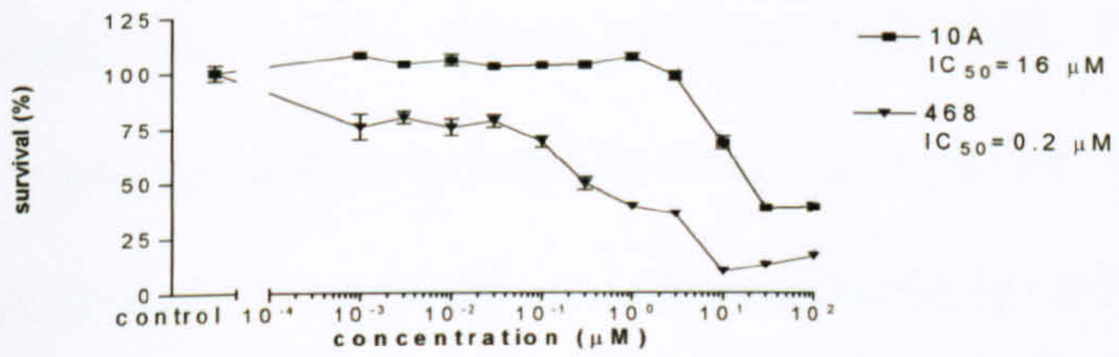

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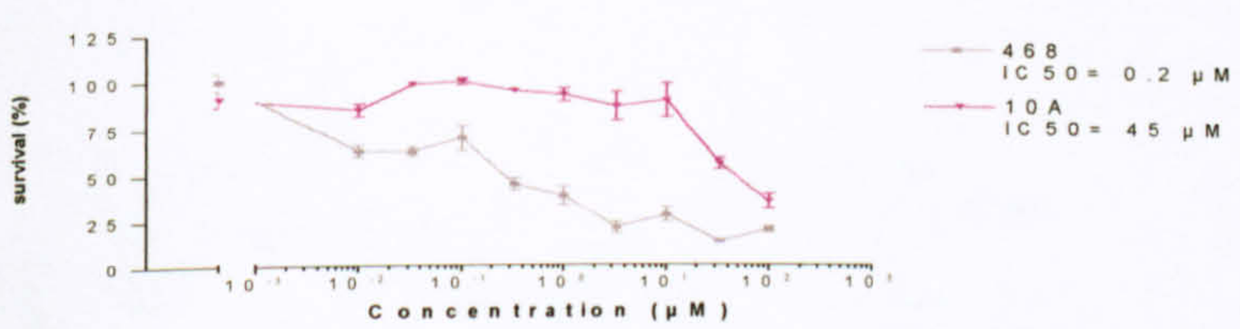

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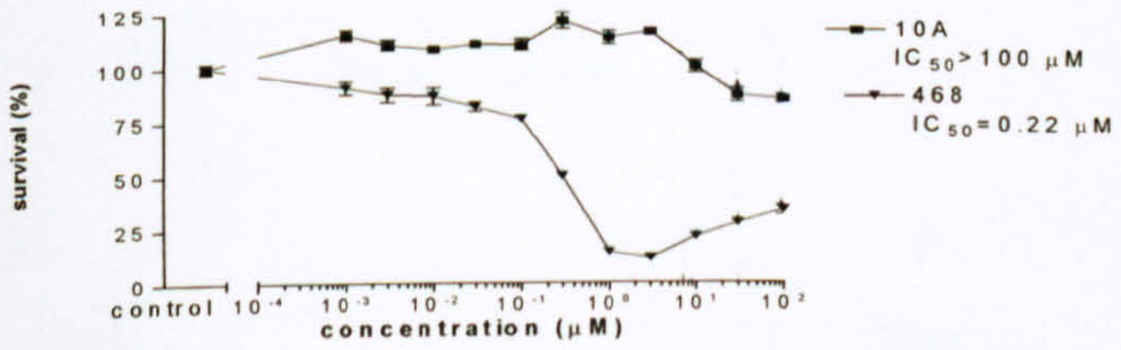
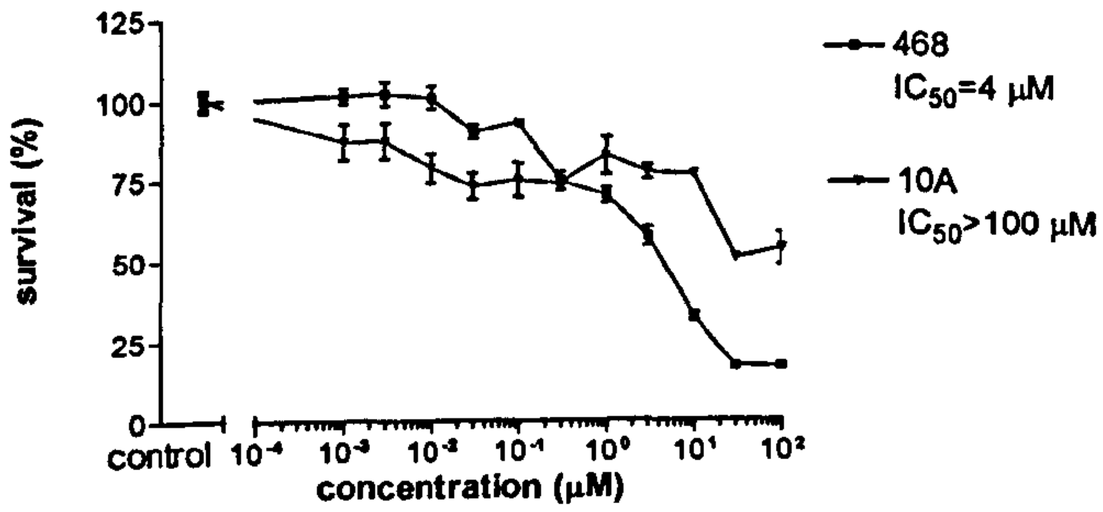


Figure 5.6 MTT assays of natural compounds on MDA-MB 468 and MCF10A cells. i) Cirsiliol ii) Eupatorin iii) Eupatorin-5-methyl ether. The IC_{50} of eupatorin was calculated as an average of eight replicates, taking into consideration the inhibitor study. The assay for cirsiliol and eupatorin-5-methyl ether was repeated at least three times. Error bars represent standard deviation of the mean.

cancer cells significantly with IC50s of 1 and 4 μM respectively (Figure 5.7). Baicalein, scutellarein, chrysin and apigenin (IC_{50} 50 μM data not shown) were less potent natural compounds in this cell line (Table 5.1). Acacetin had no effect on the growth of MDA-MB 468 cells (data not shown). In MCF10A cells all the compounds except baicalein and cirsiliol had IC_{50} greater than 20 μM (Table 5.1, apigenin IC_{50} >100 μM, data not shown). Baicalein and cirsiliol showed IC_{50} of 14 and 18 μM respectively (Table 5.1). Sinensetin did not exhibit an IC_{50} even at concentrations of 100 μM (data not shown). The difference in IC_{50} values, between MDA-MB 468 and MCF10A cells, in the case of chrysin, scutellarein and baicalein was analysed statistically by Students T-test and found to be significant in the case of scutellarein ($p= 0.0002$) , baicalein ($p=0.02<0.05$) and non significant in the case of chrysin ($p=0.8$).

i)



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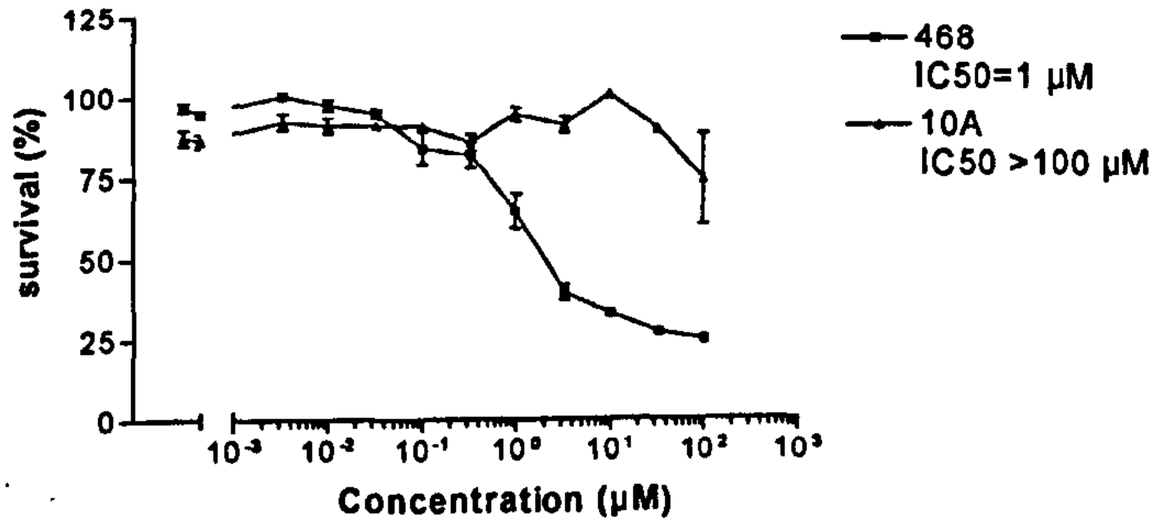


Figure 5.7 Bioactivation of natural products in MDA-MB 468 cells. i) Diosmetin ii) Genkwanin. Error bars represent standard deviation of the mean for at least n=3 replicates.

Table 5.1 The effect of dietary flavonoids on the cell viability of MCF7 (a), MDA-MB 468 (b) and MCF10A (b) cells. MCF7 cells were treated with 10 nM TCDD for 24 hr to induce CYP1 enzymes. Results are expressed as IC_{50s}, calculated from percentage of cell viability of cells treated with various concentrations of flavonoids (see chapter 2) compared to control (0.1% DMSO treated). Each value corresponds to mean ± standard deviation for at least n = 3 determinations. IC_{50s} are in µM. Significant differences were determined using Students T-test.

(a)

Compound	Induced	Non induced
Genkwanin	13.7 ± 2.3*	17.3 ± 2.5*
Eupatorin	7 ± 0*	6.9 ± 0.3*
Cirsiliol	5.3 ± 0.5**	9.5 ± 1**
Baicalein	16.7 ± 2.9*	19.3 ± 0.6*
Chrysin	60 ± 17.3*	73.3 ± 5.8*
Scutellarein	16.3 ± 3.5*	18.5 ± 1.3*
Diosmetin	11.3 ± 2.3**	20 ± 0**
Apigenin	36.7 ± 2.9*	35 ± 5*

Table 5.1 Continued

(b)

Compound	MDA-MB 468	MCF10A
Chrysin	24.5 ± 5.5*	25.3 ± 3. 7*
Baicalein	7.1 ± 0.3**	15.3 ± 3.8**
Scutellarein	12.8 ± 1.5**	41.8 ± 2.9**

* Differences were non significant ($p>0.05$)
**Differences were significant ($p<0.05$)

In order to confirm that the above effects in MDA-MB 468 cells were due to metabolism of the natural compounds from CYP1 family enzymes, MTT assays were carried out, using eupatorin and genkwanin as bioactivating compounds and acacetin as an inhibitor of CYP1 family enzymes, to find out whether that would reverse the inhibition of cell growth seen in the cases of the above compounds. Cotreatment of acacetin (2 μM) and genkwanin or acacetin and eupatorin reversed the IC_{50} from 1 μM to 10 μM and from 0.6 μM to 15 μM, respectively (Figure 5.9)

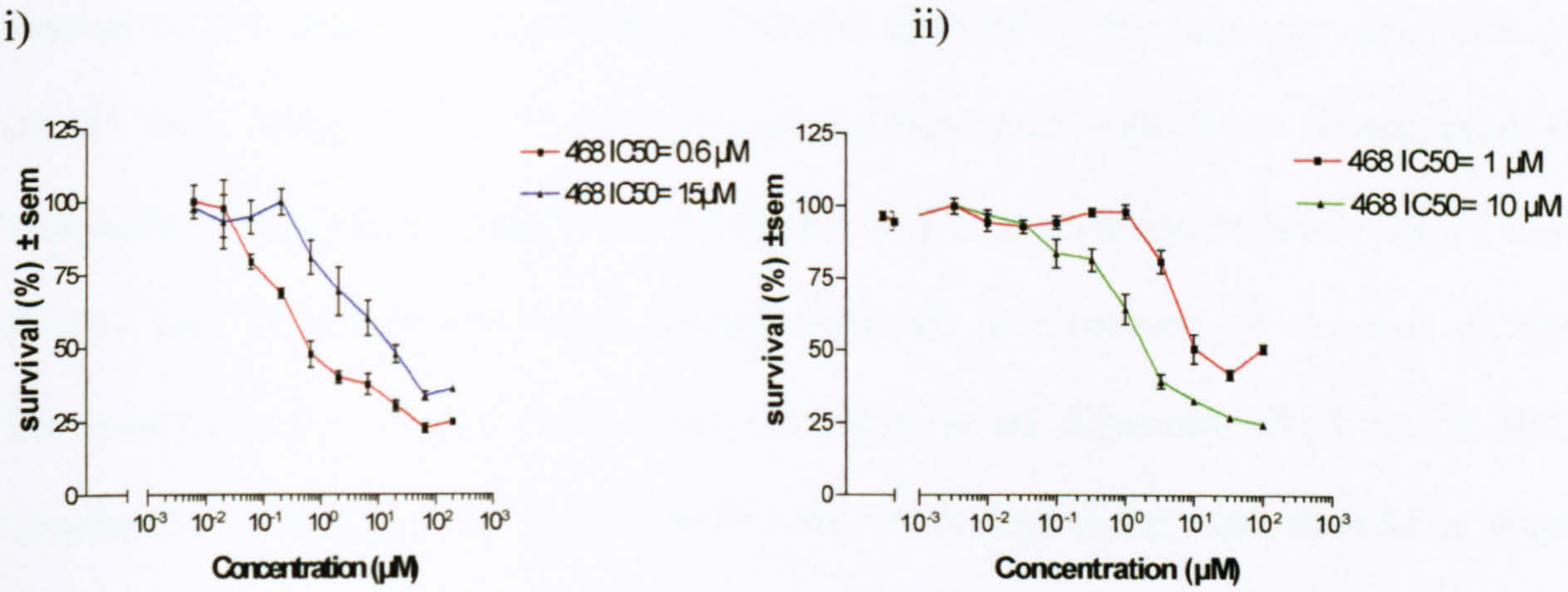
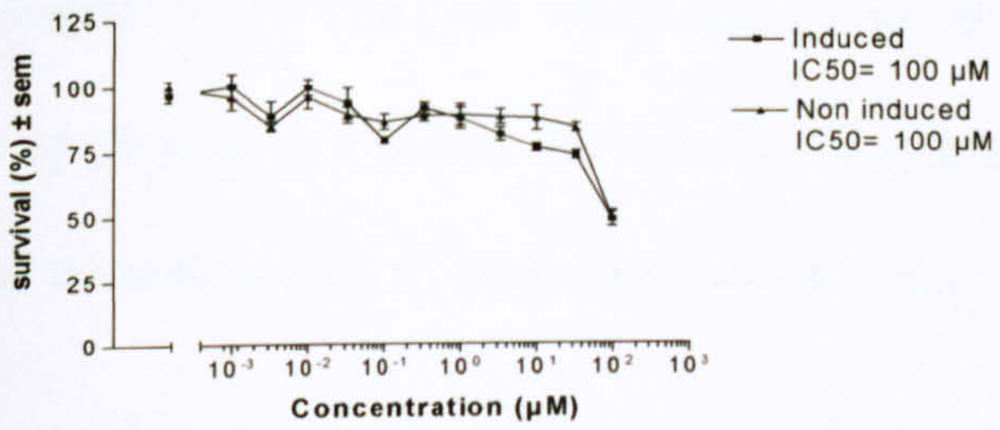


Figure 5.8 The cytotoxic effect caused by eupatorin and genkwanin in MDA-MB 468 cells is reversed with the addition of 2 μM of the CYP1 family inhibitor acacetin. i) Eupatorin; red line; eupatorin alone, blue line; eupatorin and acacetin. ii) Genkwanin; green line; genkwanin alone, red line; genkwanin and acacetin. Error bars represent standard deviation of the mean for n=3 replicates.

Apart from the above two cell lines MCF7 cells were used as a second screening model to investigate bioactivation of natural products in tumour cells. MCF7 cells are known from previous studies to be inducible (Dohr et al., 1995) from PAHs. TCDD for 24 hr, prior to compound treatment, at a concentration of 10 nM was used, to induce CYP1 enzyme expression in the above cell line and then cell viability was assessed using the MTT assay as described before. The efficiency of CYP1 enzyme expression after induction with TCDD, was checked periodically every 2 months, by EROD assay and HPLC metabolic profiles of flavones.

The most potent compounds in this cell line model were cirsiliol (IC_{50} 5.25 ± 0.5 μM, Table 5.1) and eupatorin (IC_{50} 7 ± 0 μM, Table 5.1). The IC_{50} value of cirsiliol in TCDD treated MCF7 cells was significantly different ($p=0.001<0.05$) than the one seen in MCF7 cells alone (9.5 ± 1 μM), whereas no difference was found in the case of eupatorin. There was no significant difference in the IC_{50} values, between MCF7 non treated and TCDD treated cells, noticed from 96 hr treatment of the rest of the flavones(Table 5.1, Figure 5.9), except in the case of diosmetin (11.3 ± 2.3 μM, compared to 20 ± 0 μM, $p=0.02<0.05$, table 5.1). Sinensetin also showed a great differential in IC_{25} values between TCDD induced (IC_{25} = 0.1 μM) and non induced MCF7 (IC_{25} > 100 μM) cells.

i)



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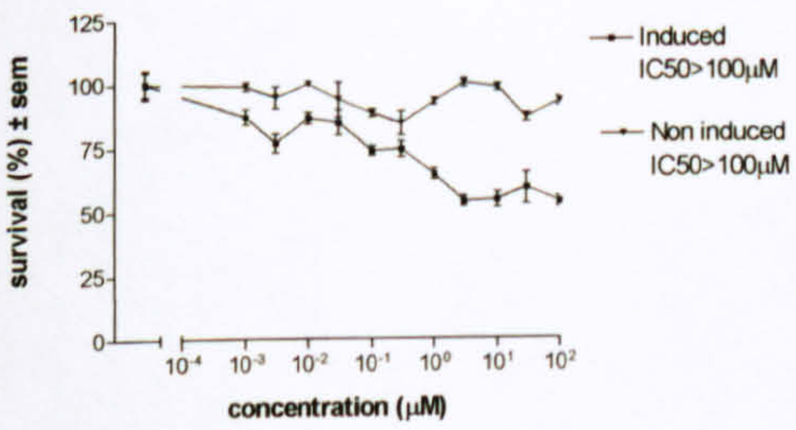
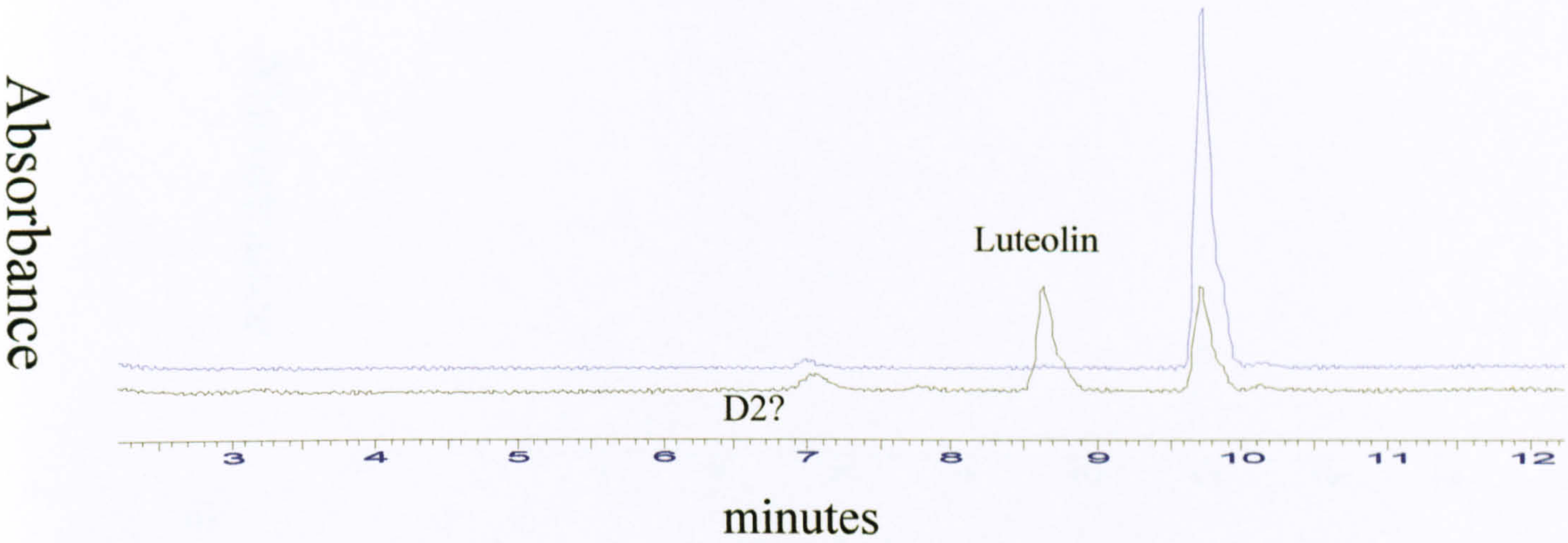


Figure 5.9 Cytotoxicity of dietary flavones in the MCF7 cell line. MCF7 cells were induced with 10 nM of TCDD for 24 hr, prior to treatment of the compounds. i) Eupatorin-5-methyl ether. ii) Sinensetin.

The metabolism of three of the above flavones in MCF7 cells preinduced with 10 nM TCDD, was also investigated. Diosmetin was metabolised to luteolin in both cells and medium extracts (Figure 5.12). No coelution studies were performed, because the polarity of diosmetin and luteolin results to a relative retention time of 1 minute as reported from the metabolism in the CYP1 microsomes. This was clearly shown from the reults obtained. A second metabolite eluted at 7 min. No metabolism was observed in MCF7 cells. Eupatorin was metabolised to three metabolites in MCF7 cells pretreated with dioxin. Unfortunately these metabolites eluted at different retention times than those observed from eupatorin metabolism in the CYP1 microsomes in chapter 4 (Figure 5.13). This difference is probably due to impurities present in the samples produced from the cells and the medium. Genkwanin metabolism produced two unidentified metabolites and (Figure 5.14). The peak which appears at 10.5 minutes corresponds to apigenin. Apigenin was present as a minor impurity in the genwanin standard (Figure 5.14). There was little or no metabolism of genkwanin to apigenin from pretreated MCF7 cells.

i)



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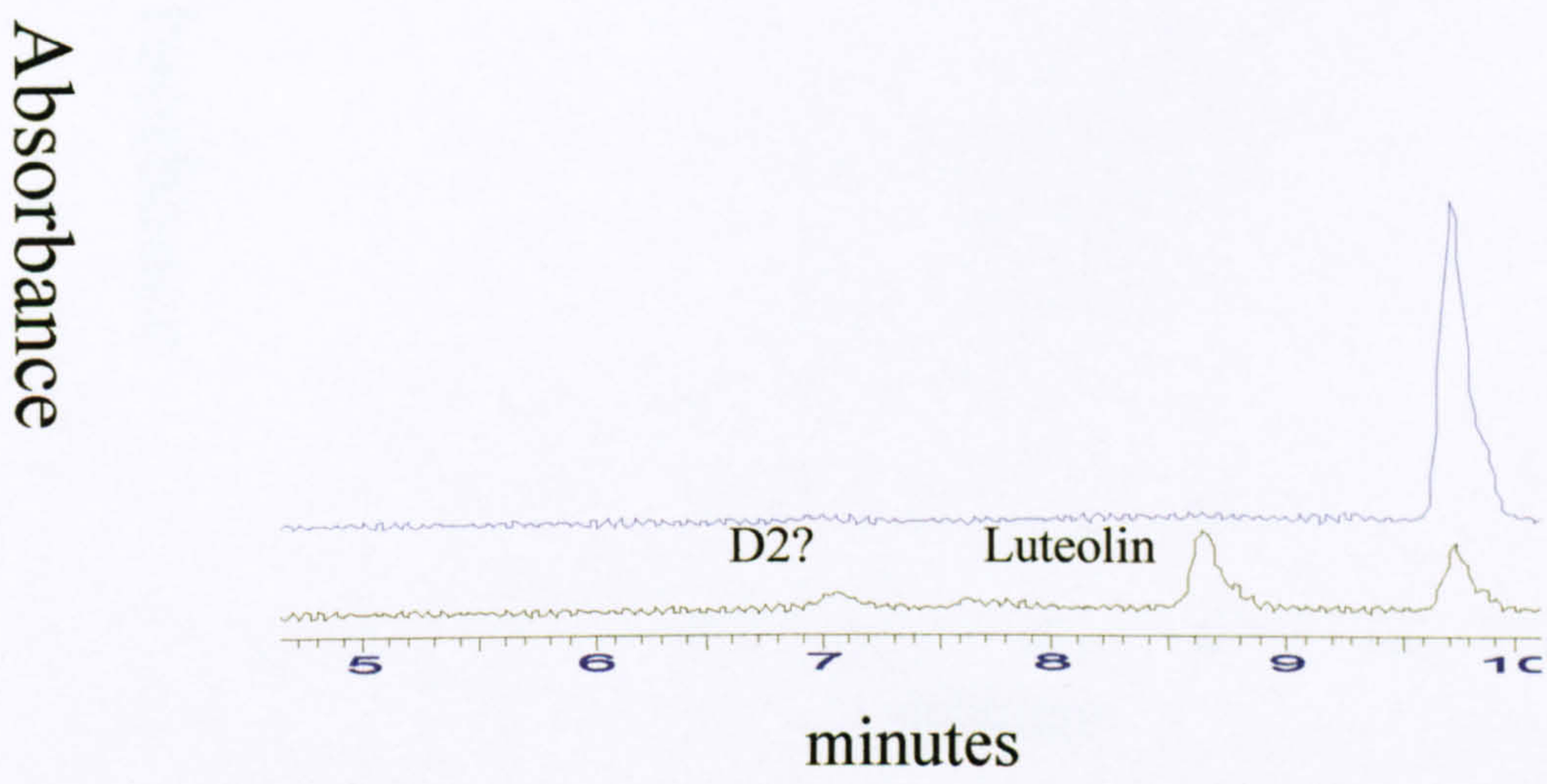


Figure 5.12 Diosmetin (10 μM) metabolism in the MCF7 induced cell model. MCF7 cells were pretreated with 10nM of TCDD to induce CYP1 enzyme expression prior to addition of the natural product. i) Medium extracts; black; MCF7 cells pretreated with dioxin, blue; MCF7 cells. ii) Cell extracts; black; MCF7 cells pretreated with dioxin, blue; MCF7 cells.

Experiments were done in duplicate.

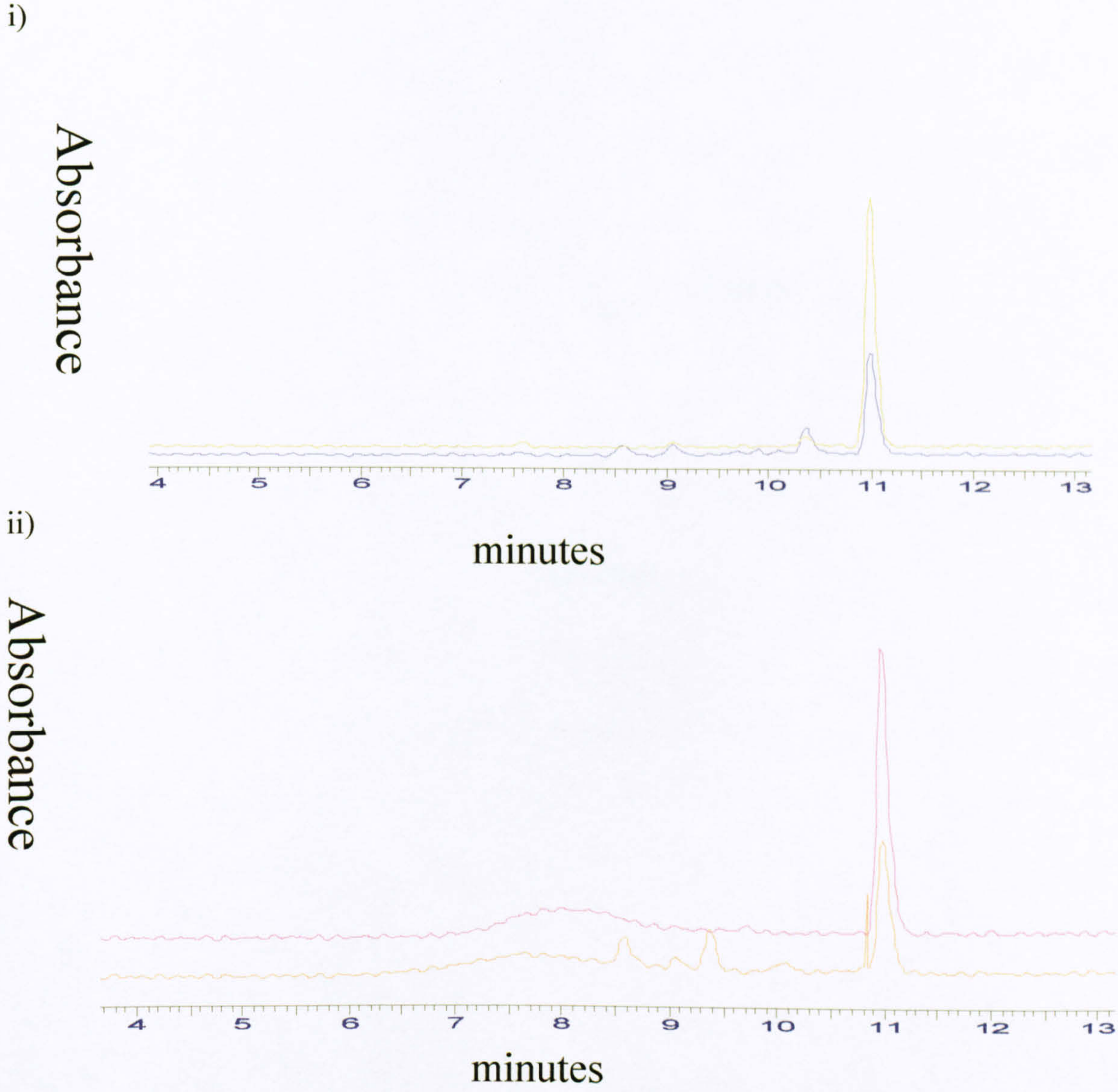
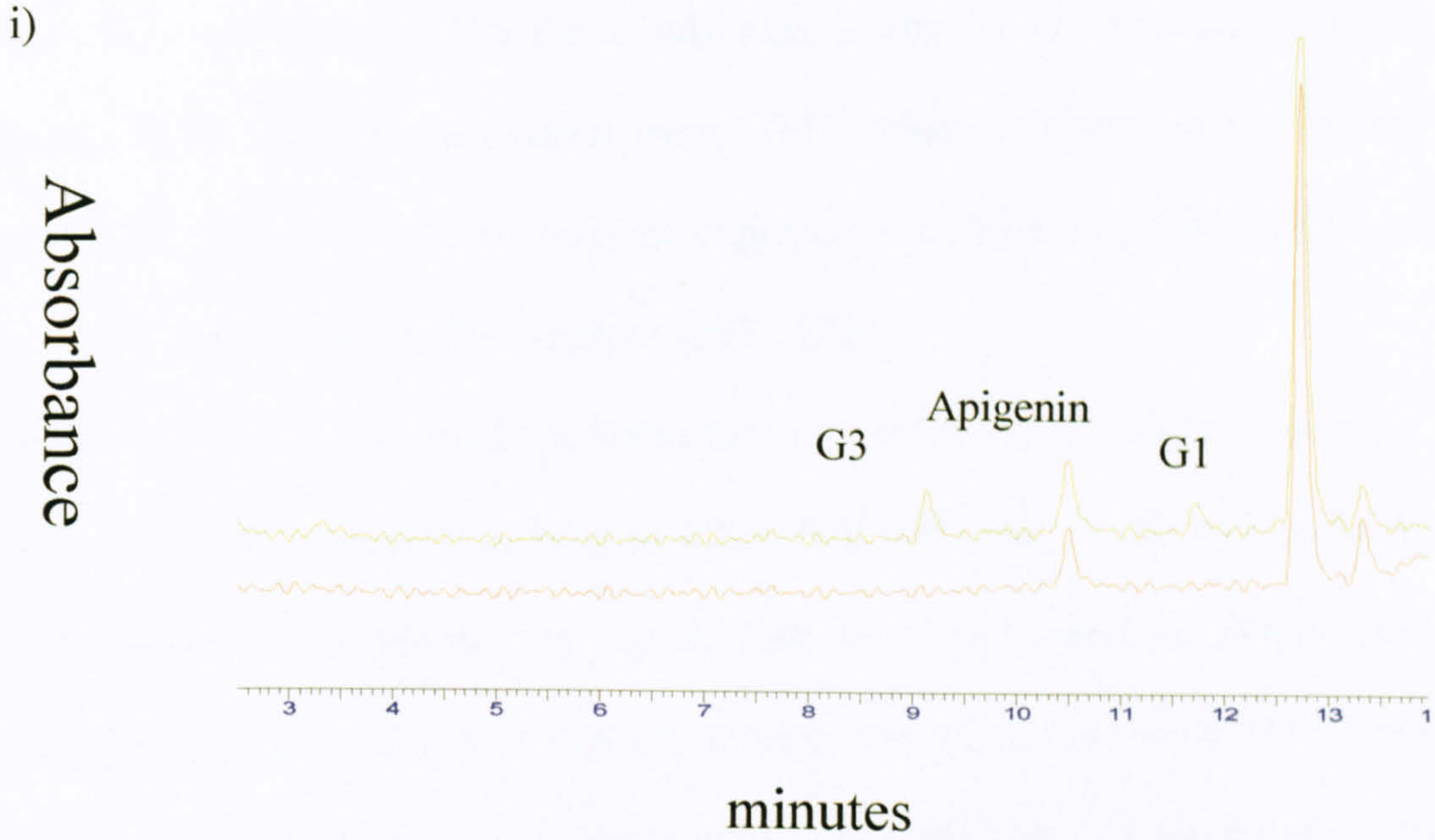


Figure 5.13 Eupatorin (10 μM) metabolism in the MCF7 induced cell model. MCF7 cells were pretreated with 10nM of TCDD to induce CYP1 enzyme expression prior to addition of the natural product. i) Medium extracts; grey; MCF7 cells, blue; MCF7 cells pretreated with dioxin. ii) Cell extracts; red; MCF7 cells, orange; MCF7 cells pretreated with dioxin.

i)



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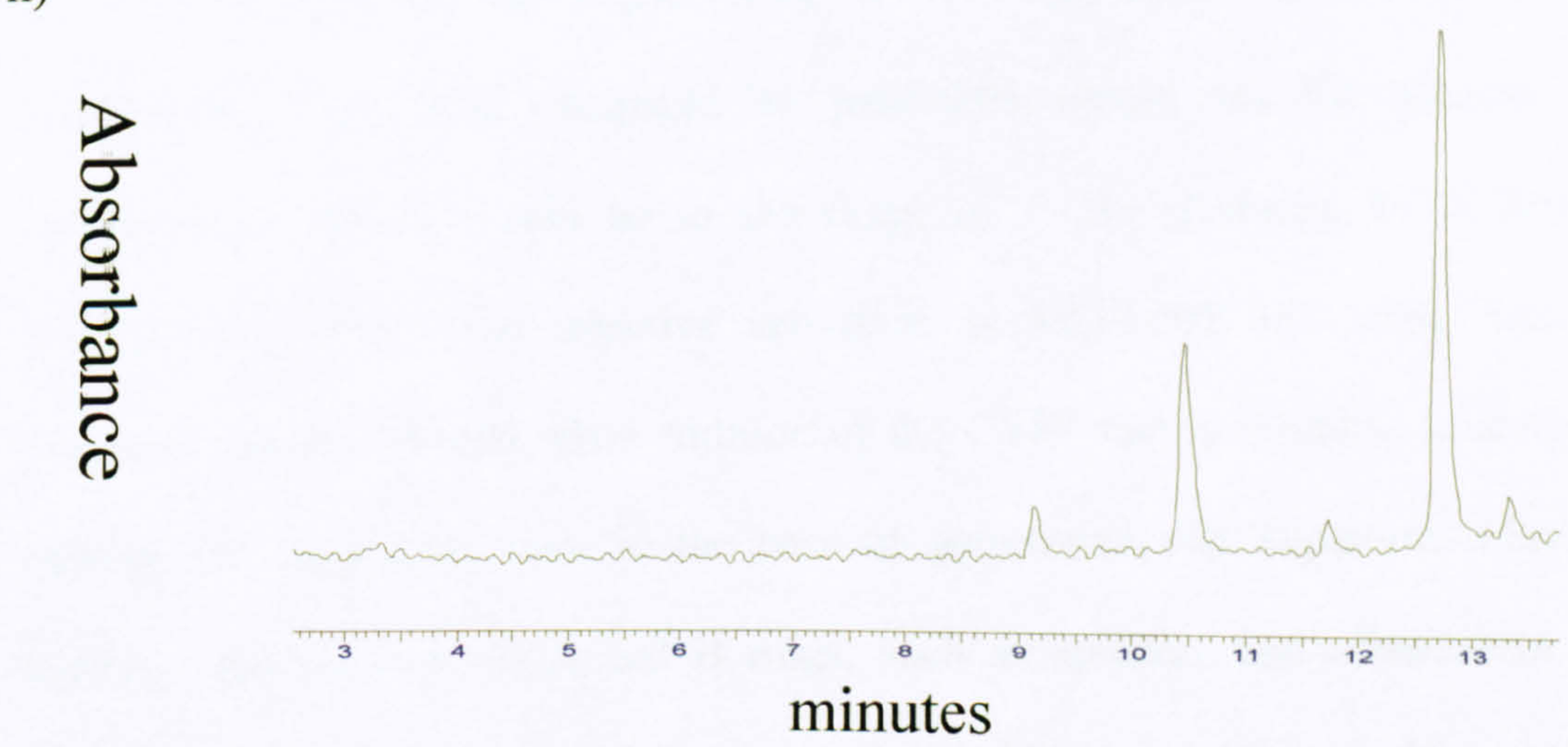


Figure 5.14 Genkwanin (10 μM) metabolism in the MCF7 induced cell model. MCF7 cells were pretreated with 10nM of TCDD to induce CYP1 enzyme expression prior to addition of the natural product. i) Medium extracts; orange; MCF7 cells, brown; MCF7 cells pretreated with dioxin. ii) Medium extracts; MCF7 pretreated with 10nM TCDD, spiked with 2 μM apigenin.

In this chapter the effects of dietary flavonoids on breast cancer cells and normal breast cells were summarised. The flavonoids were shown to be cytotoxic in MDA-MB 468 cells, due to metabolic activation from CYP1 family enzymes and a lot less toxic in MCF10A cells. CYP1 family enzyme expression in MDA-MB 468 cells was confirmed by EROD activity and HPLC analysis (p129,130).

With regard to flavonoid structure-activity relationship, some structural features associated with cytotoxic activity in MDA-MB 468 cells could be identified. Flavones with methoxy substituents on the A ring such as sinensetin, genkwanin, cirsiliol, eupatorin and eupatorin-5-methyl ether were very potent to affect cell viability in this model. This toxicity seen was due to bioactivation from CYP1 family enzymes, because the same compounds had higher IC_{50s} in MCF10A cells. All the compounds had considerably high IC_{50} compared to published results on the plasma flavonoid concentration, which would be in the range of 1 μM (Kuhnau, 1976, Scalbert and Williamson, 2000). This selective activation in MDA-MB-468 cells, due to CYP1 enzymes was also shown when addition of the CYP1 family inhibitor acacetin reversed partially the cytoxicity seen in the case of genkwanin and eupatorin. Flavones with hydroxy substitutions on A and B rings, such as apigenin and scutellarein were less potent, but still showed a small activation factor. Flavones with no hydroxy substitutions on the B ring such as chrysin and baicalein were of intermediate efficacies, but this effect was probably not due to bioactivation from CYP1 family enzymes. Chrysin was a poor substrate for CYP1 family enzymes and baicalein was not metabolised at all as shown in chapter 4, which is in agreement with the MTT results, showing no major difference between MDA-MB 468 and MCF10A cells.

The metabolic profiles of the flavones investigated, observed in MDA-MB 468 cells, were similar to those seen in the enzyme assay based system in chapter 4. Comparison of the IC_{50s} obtained for diosmetin and luteolin in MDA-MB 468 (1.4 μM data not shown) and MCF10A (42 μM data not shown) cells, leads to the conclusion that the bioactivation observed in the case of diosmetin is probably mainly due to the second metabolite (D2 or 6-hydroxydiosmetin) which elutes at 7 min. Luteolin was produced in small amounts and did not appear to be that toxic in MCF10A cells. Genkwanin also produced no apigenin in the above cell lines as opposed to the enzyme assay. The cytotoxic action of this flavone is probably due to metabolism on a different part of the ring than the 7 position. It is possible that the unidentified metabolite G1, which could be either 6-hydroxy or 3′-hydroxygenkwanin is causing the observed cytoxicity in the breast cancer cells. The cytotoxicity seen in the case of eupatorin was partially due to cirsiliol and due to E1, as noted from the IC50s of these two compounds on both normal and cancer cells.

In MCF7 cells, pretreated with 10 nM TCDD, all the flavones exhibited little or no bioactivation compared to MCF7 cells alone. Initially it was believed that this was because no metabolism was occuring. However this was proven not to be the case. HPLC analysis showed similar metabolic patterns to those seen in the enzyme assay. Moreover sinensetin exhibited an unusual pattern of activation in TCDD treated MCF7 cells. It seemed as if the compound was activated to more cytotoxic species, but this was not enough to reduce the IC_{50} to a lower value compared to the one seen in the non induced cells. By comparing the IC_{25} values though, a 1000 fold activation factor was seen between the induced and non induced cells. Taken together these data suggest that the induction of CYP1 family enzyme expression, by TCDD in MCF7 cells, is transient and

not maintained through the whole 96 hr exposure to the compounds, as opposed to MDA-MB 468 cells, where the enzymes are constitutively expressed. Thus these two cell line models cannot be used as a comparison.

Diosmetin was metabolised in both MDA-MB 468 and MCF7 cells preinduced with dioxin. The amount of luteolin formed in MCF7 cells was a lot higher than the one seen in MDA-MB 468 cells. In chapter 4 it was shown, that luteolin is preferentially formed from diosmetin metabolism from CYP1A1. Hence it can be speculated that MCF7 cells preinduced with dioxin express mainly CYP1A1 in the active form. Moreover D1 (6-hydroxydiosmetin) was seen in both MDA-MB 468 and MCF7 cellular metabolism. The amount of that metabolite seemed to be higher in MDA-MB 468 cells, compared to the formation luteolin. CYP1B1 in the enzyme assay system did not produce any D1. This probably means that MDA-MB 468 cells express very little CYP1B1.

Previous work in the literature has underlined the importance of flavonoids as chemopreventative agents by inhibition of cell growth in several cancer cell lines (Kawaii et al., 1999). Quercetin, luteolin and apigenin are from the most commonly encountered flavonoids in the literature. Apigenin, luteolin and chrysin have been shown to inhibit MCF7 cell proliferation at high concentrations close to 50 μM (Le Bail et al., 1998). In another study (Guthrie et al., 1997) apigenin exhibited an IC_{50} of 2.4 μg/ml and 3 μg/ml in MCF7 and MDA-MB 435 respectively, which is close to 10 μM. Baicalein and apigenin have been shown (Sonoda et al., 2004) to significantly inhibit cell growth of HL-60 cells with IC_{50s} of 23 and 15 μM respectively. Luteolin and quercetin were shown to inhibit cell growth of HT29 colon adenocarcinoma cells with IC_{50s} of 30 and 15 μM respectively (Agullo et al., 1996). In the same study apigenin had similar effect to

luteolin and diosmetin was less potent than both of these flavones. In a recent study a resveratrol analogue 3,4,5,4′-tetramethoxystilbene was shown to be a lot more cytotoxic in cancer cells (W138VA, IMR-90SV, LNCap, Hela, HT-29 and HepG2) than normal cells (W138, IMR-90, BJ-T) (Gosslau et al., 2005). The same molecule selectively induced apoptosis at low levels in the transformed W138VA cancer cell line, whereas resveratrol induced apoptosis in the same cells at very high concentrations (Gosslau et al., 2005).

It is important to note that all the above studies have examined the intrinsic cytotoxic effects of the flavonoids on several cancer cell lines. This is the reason why the IC_{50s} of the flavonoids observed in such studies are a lot higher than those seen in MDA-MB 468 cells. The results presented here, show that biotransformation of dietary flavonoids in cancer cells is possible, and can yield highly active metabolites, which can cause selective inhibition of cell growth. The increase in activation seemed to be dependent on the methoxy groups present in A and B rings of the flavonoids. Generally the more methoxy groups were present the greater the activation, since they could undergo multiple demethylation reactions by CYP1 family enzymes. Increase in hydroxylation substitutions increases the reactivity of the flavonoids, e.g. quercetin is more reactive than kaempferol, because the two hydroxyl groups present on the B ring makes the molecule more susceptible to oxidation than kaempferol. Indeed quercetin has been shown to have higher mutagenic activity than its hydroxylated flavone equivalents and kaempferol in TA 98 strain in *in vitro* microbial mutagenic assays (Middleton et al., 2000). The presence of the 3 and 3′ hydroxyl group causes quercetin to be oxidised rapidly to quinone reactive intermediates, which cause DNA damage in these type of

assays. Moreover mutagenic studies *in vivo* have shown that methylation of free hydroxyl groups of flavonoids by catechol-*O*-methyltransferase (COMT) would significantly reduce their mutagenicity (Middleton et al., 2000). Further hydroxyl groups on either the A or the B ring of the flavones could interact with key signalling molecules in the cell. Piceattanol has already been shown to be a tyrosine kinase inhibitor and CYP1B1 can produce it from resveratrol (Potter et al., 2002).

Furthermore this is the first study ever to show, that natural products such as diosmetin and eupatorin can be metabolised in cancer cells by cytochrome P450s, such as CYP1A1 and CYP1B1, which were earlier believed to promote carcinogenesis through conversion of PAHs to their ultimate carcinogens. In this regard CYP1A1 and CYP1B1 can be termed as "rescue enzymes" or tumour suppresor enzymes, which can inhibit tumor cell proliferation through metabolic activation of circulating natural products.

However in order for such molecules to exert their biological activity, they must fit the substrate specificity criteria discussed earlier in chapter 4. All the flavonoids examined in these studies could be overlayed structurally upon oestradiol, which is the endogenous substrate of CYP1B1. Furthermore the double bond between C_2 and C_3 in the C ring of the flavonoid structure seems to make the molecules more rigid and thereby enhancing their capacity to enter the active sites of CYP1 family enzymes. These results provide more insight to the chemopreventative action of this class of natural products.

CHAPTER 6. EFFECTS OF FLAVONOIDS ON THE CELL CYCLE OF TUMOUR AND NORMAL CELLS

6.1 Introduction

The phenomenon of apoptosis or programmed cell death has been reviewed in the past years (Jacobson and McCarthy, 2002). Some anticancer drugs have been shown to cause apoptosis in cancer cells (). Flavonoids can cause apoptosis in tumor derived cell lines e.g. tangeretin (5,6,7,8,4′-pentamethoxyflavone) induced apoptosis in HL-60 cells at concentrations greater than 2.7 μM (Middleton et al., 2000).

In addition flavonoids with structural features such as piceattanol, which is a tyrosine kinase inhibitor, can potentially inhibit the progression of cells through the phases of the cell cycle. It is also possible that flavonoids can directly inhibit the phosphorylating kinase activity of the cyclin-CDK complexes as shown by the synthetic flavopyridol (section 1.1.6.2 Figure 1.14), which resembles the flavone structure.

Based on the above evidence and having previously shown in chapter 5 that the flavones examined inhibited the growth of MCF7 and MDA-MB 468 cells, due to endogenous activation from CYP1 enzymes, it was investigated, whether the inhibition of cell growth was due to apoptosis or cell cycle arrest, using flow cytometric DNA analysis. The flavones investigated in this type of assay are shown in Figure 6.1. Methoxylated flavones were the main focus, because they had previously shown low IC_{50} values in MDA-MB 468 cells.

6.1.1 Introduction to flow cytometry

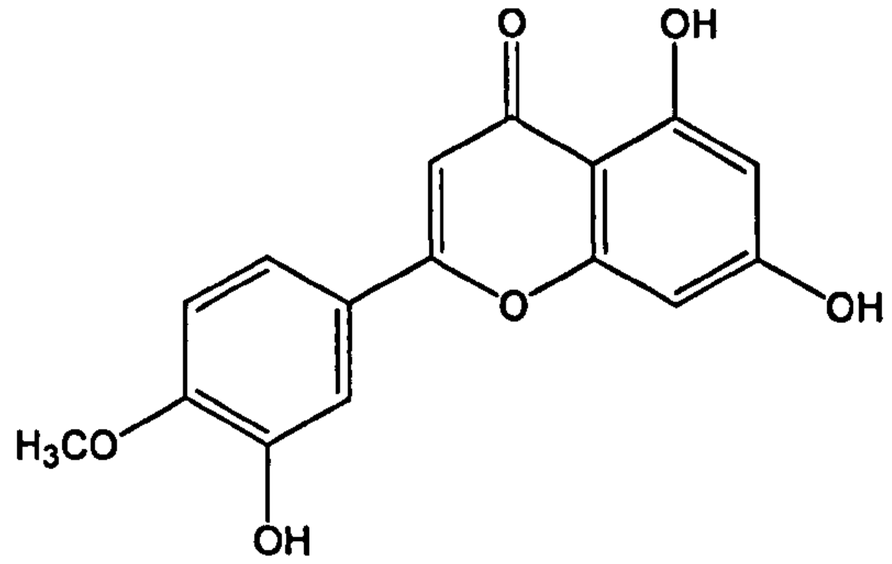
Flow cytometric analysis has been extensively used for the quantitation of DNA in tumour cells for both research and clinical purposes. Substantial loss or gain of DNA has been shown in the genome of neoplastic cells by flow cytometric analysis. These changes

are characterised as aneuploidy and have been used as diagnostic and prognostic markers in a variety of solid tumours. Aneuploidy is a chromosomal state in which abnormal numbers of specific chromosomes or chromosome sets exist within the nucleus(www.linguasphere.org/dictionary/n-2976-aneuploidy.html).

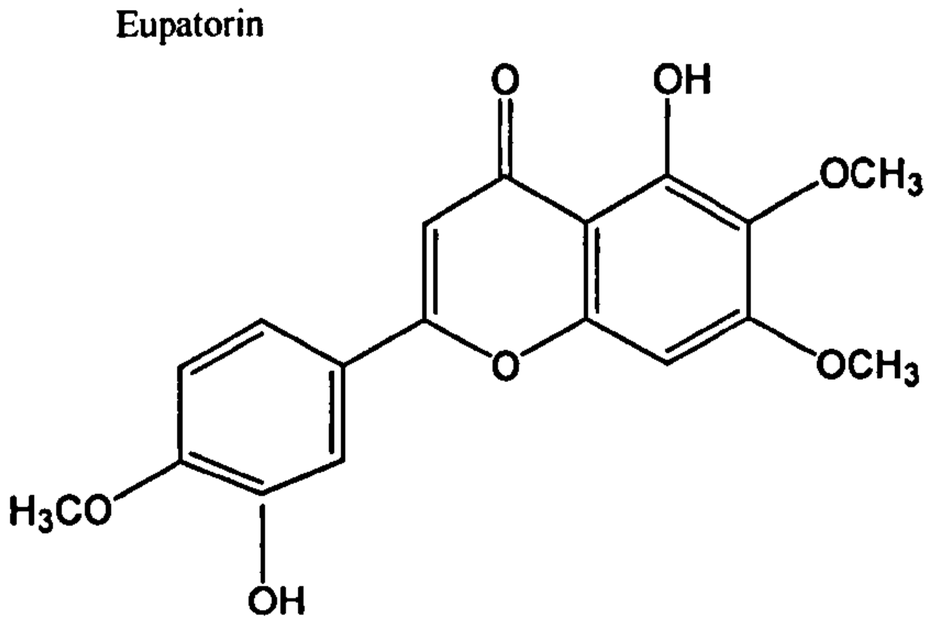
Moreover flow cytometry has been applied to the study of cell cycle analysis and apoptosis in cancer research. It has been proven to be an invaluable tool, which provides information about the mechanism of action of various chemotherapeutic agents. Flow cytometry overcomes the limitations of microscopy, by allowing the analysis of a large number of cells in a very short time. Microscopic studies can be used for the analysis of individual cells, however the limited number of cells observed in such studies precludes the quantitative cell analysis of entire populations.

A DNA histogram is obtained when cells are stained with a fluorescing compound, that binds quantitavely to DNA and the relative fluoresence per cell is analysed for a number of cells (Hughes and Mehmet, 2003). In most normal cell populations the majority of the cells are in G_0/G_1 phase of the cell cycle. These cells have a normal diploid (2n) DNA content. The remaining cells of the population can be seen in S and G_2/M phases. Cells which are in G_2 or M phase have a tetraploid (4n) DNA content and cells in the S phase have DNA content between 2n and 4n. FACS instrumentation is described in more detail in chapter 2 (section 2.11).

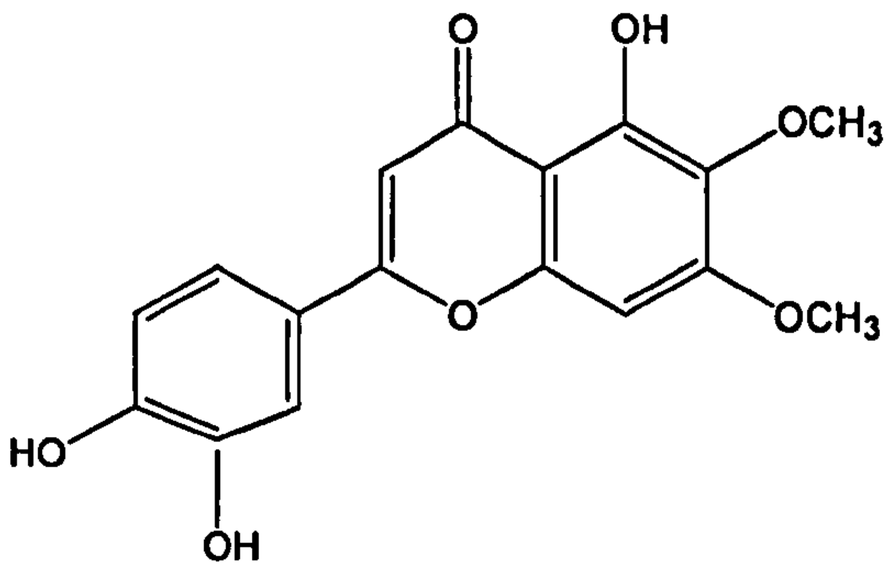
Diosmetin



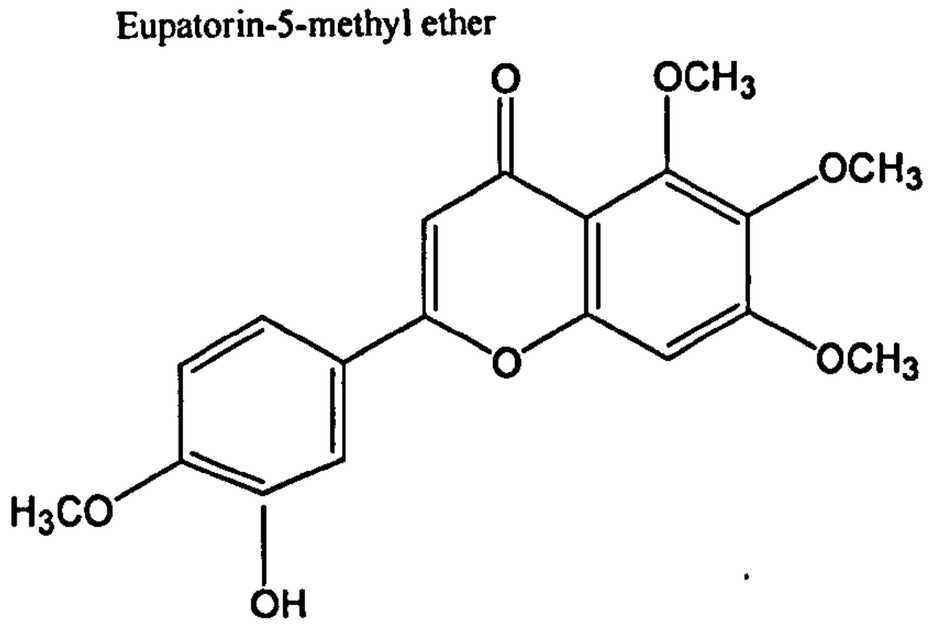
Eupatorin



Cirsiliol



Eupatorin-5-methyl ether



Sinensetin

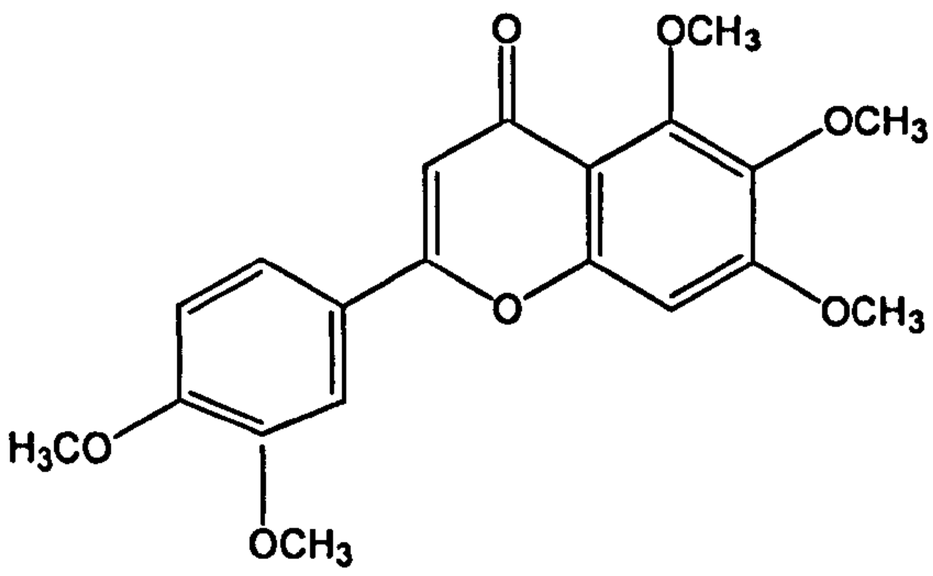


Figure 6.1 Structural features of the flavones investigated by flow cytometry

6.2 Results and discussion

The effect of two flavones eupatorin and eupatorin-5-methyl ether was investigated on the cell cycle progression of MCF7 cells. No subG_1 peak was detected in all the samples tested. At 30 μM eupatorin and eupatorin-5-methyl ether induced a small G_1 arrest in normal and dioxin treated MCF7 cells (Figure 6.2). The effect was augmented in the dioxin treated cells. Both compounds were of similar potency. There was no major effect of the compounds on the cell cycle of the MCF7 cells (control) even at very high concentrations such as 50 μM (data not shown).

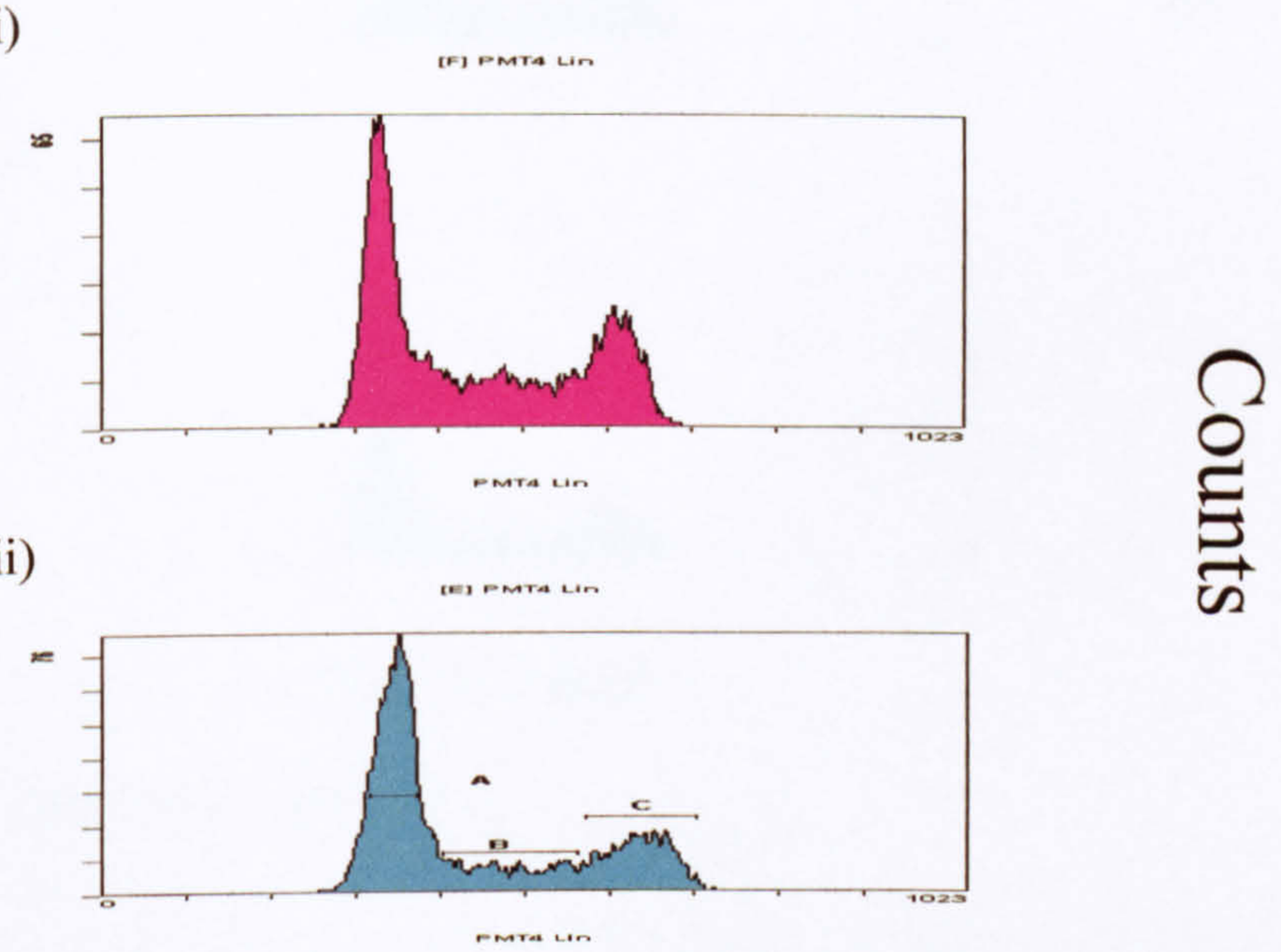
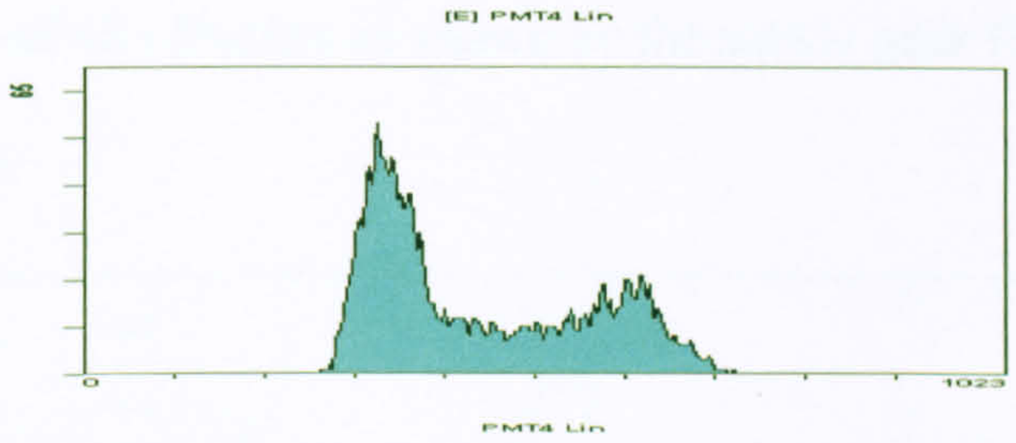


Figure 6.2 Cell cycle histograms of MCF7 cells (induced and non induced) treated with high concentrations of eupatorin and eupatorin-5-ether. i) MCF7 control (0.1% DMSO); G_1 48.8%, S 22.3%, G_2 28.9%. ii) MCF7 pretreated with 30 μM eupatorin for 24 hr; G_1 53%, S 22%, G_2 25%. iii) MCF7 (induced with 10 nM TCDD) pretreated with 30 μM eupatorin for 24 hr; G_1 60%, S 22.6%, G_2 17.4%. iv) MCF7 pretreated with 30 μM eupatorin-5-methyl ether for 24 hr; G_1 53%, S 24%, G_2 23%. v) MCF7 (induced with 10 nM TCDD) pretreated with 30 μM eupatorin-5-methyl ether G_1 60%, S 20%, G_2 20%.

iii)



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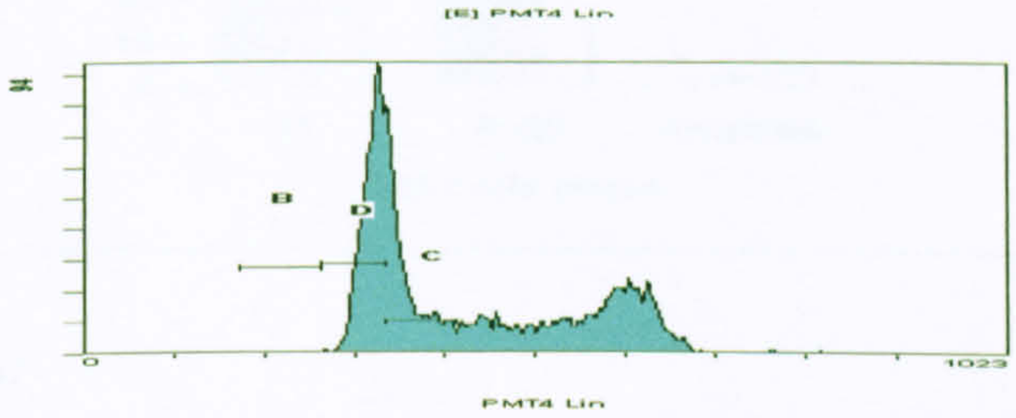
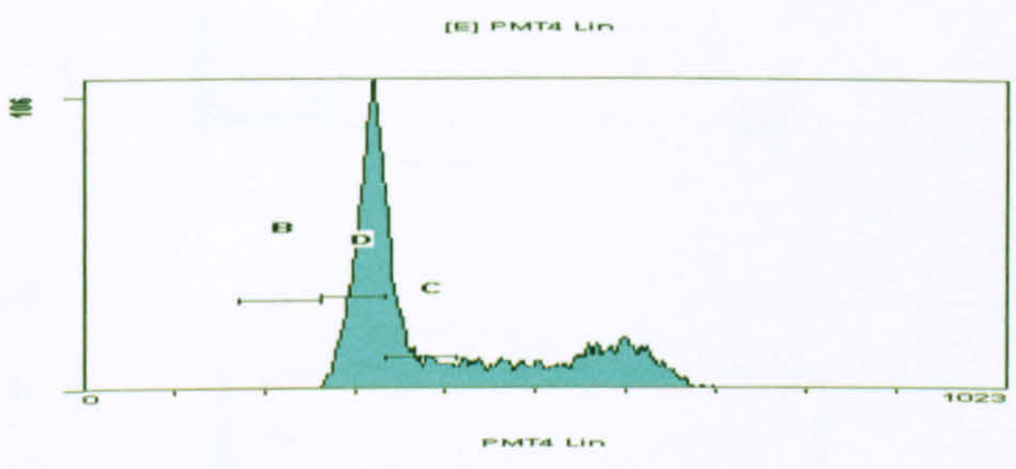


v)



Counts

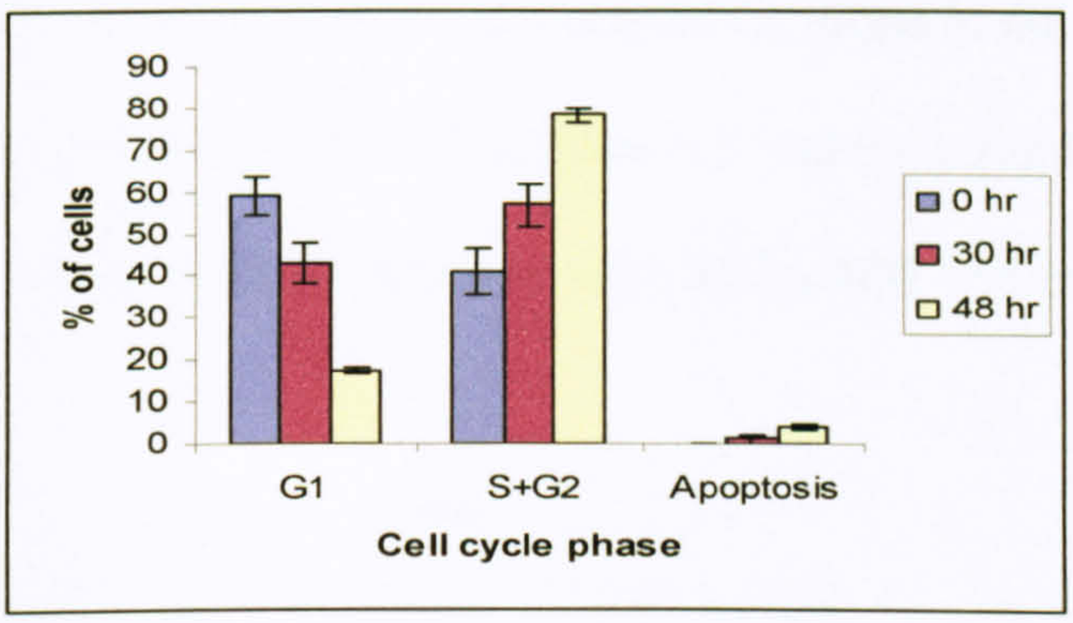
DNA Content

Figure 6.2 Continued

Several compounds were investigated at 10 μM concentration in MDA-MB 468 and MCF10A cells. The incubation time was extended to 30 hrs and if no significant effect was observed a second experiment was conducted at 48 hrs for some of the compounds. Eupatorin was the most active compound in this type of assay. At 30 and 48 hrs eupatorin arrested MDA-MB-468 cells in the G_2/M phase of the cell cycle (Figure 6.3). The results were analysed by students T test and found to be significant for the 30 ($p=0.02<0.05$) and

the 48 ($p=0.004<0.05$) hr period, respectively. Apoptosis was also observed after 30 (2%) and 48 (4%) hrs as shown by the $subG_1$ peak (Figure 6.3).

i)



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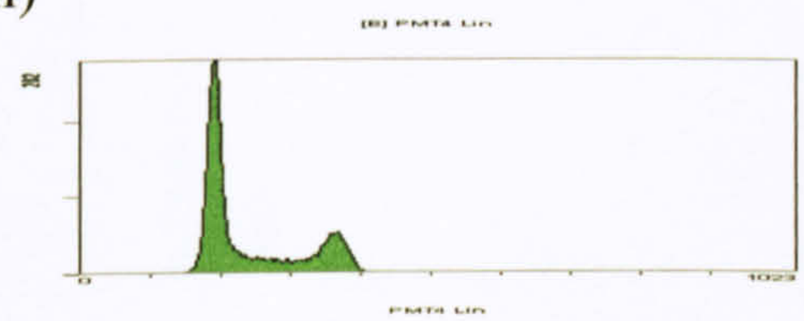

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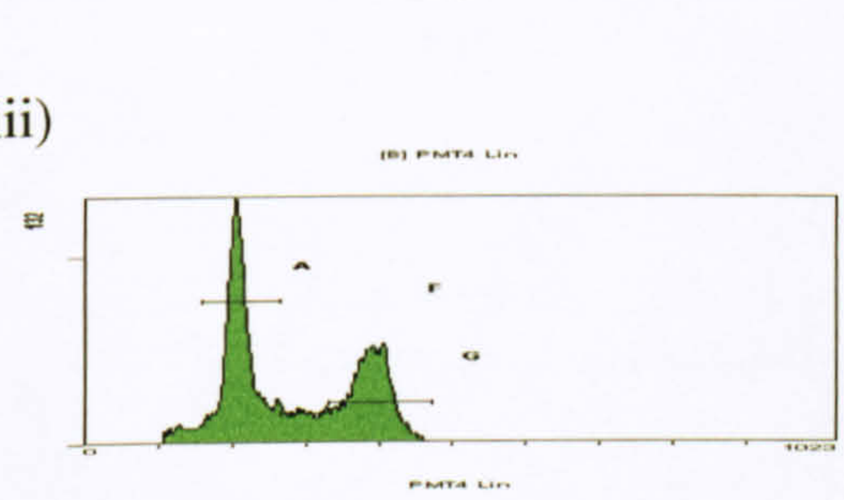

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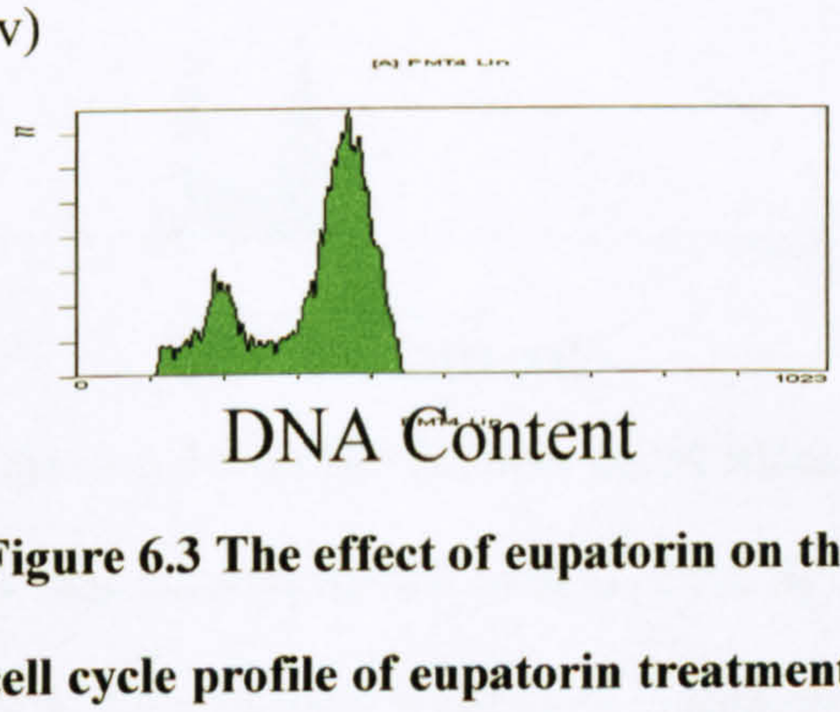


Counts

DNA Content

Figure 6.3 The effect of eupatorin on the cell cycle of MDA-MB 468 cells. i) Summary of the cell cycle profile of eupatorin treatment on MDA-MB-468 cells. Error bars represent Stdev for n=3 determinations. ii) Control; 0.1% DMSO 48 hr exposure; G_1 61% S 15% G_2 24% iii) Eupatorin (10 µM) 30 hr exposure; $subG_1$ 2% G_1 46% S 16% G_2 36% iv) Eupatorin (10 µM) 48 hr exposure; $subG_1$ 5% G_1 17% S 8% G_2 70%.

Cirsiliol at 48 hrs caused an increase of MDA-MB 468 cells present in the G_2/M phase (Figure 6.4), while at 30 hr treatment it did not have any major effect (data not shown). Sinensetin and diosmetin caused G_1 arrest in the above cell line at the 30 hr period and 48 hr period respectively (Figure 6.5 and 6.6). Eupatorin-5-methyl ether had no major effect even after 30 hr treatment with MDA-MB 468 cells at 5 μM (data not shown).

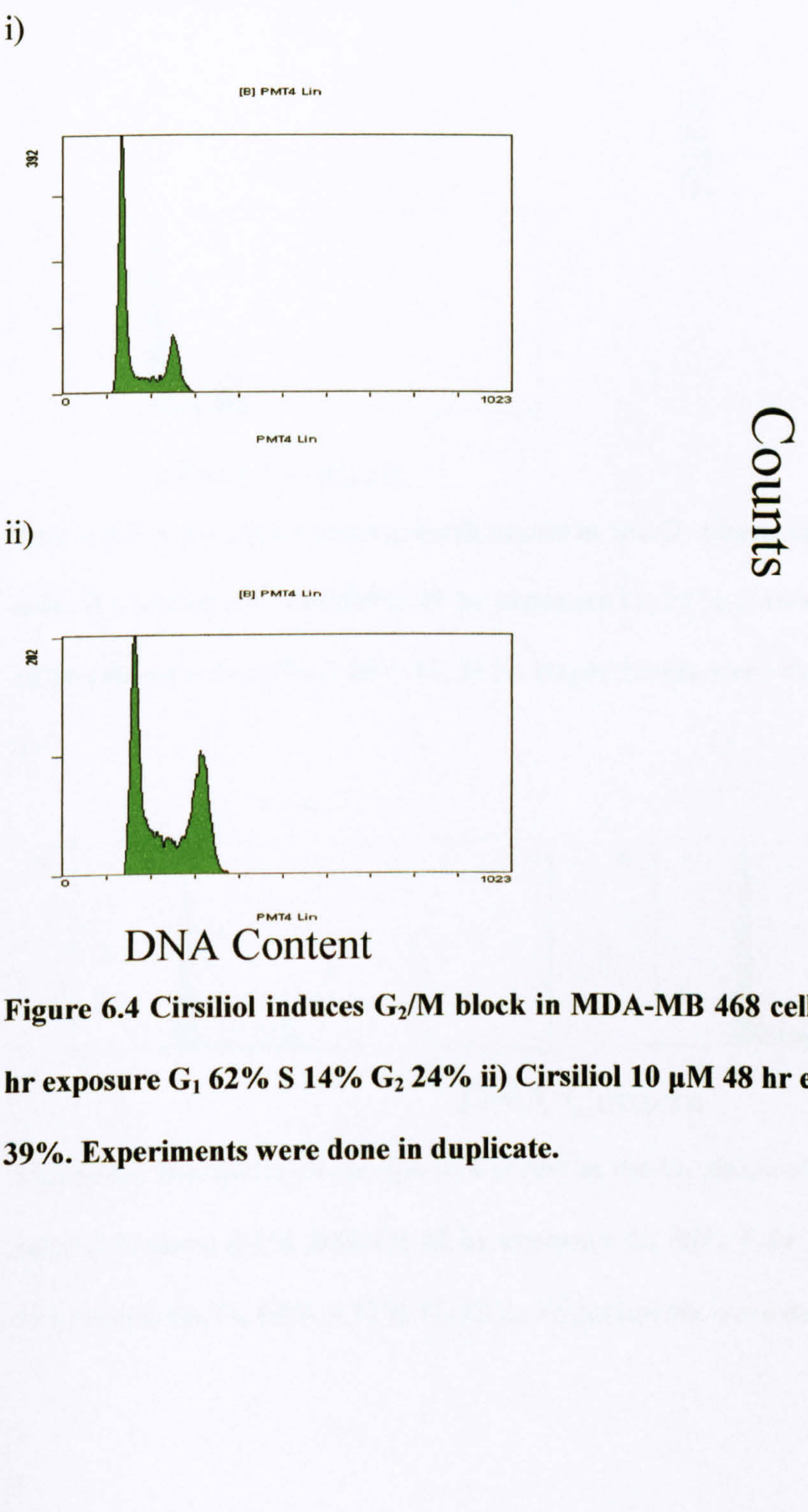
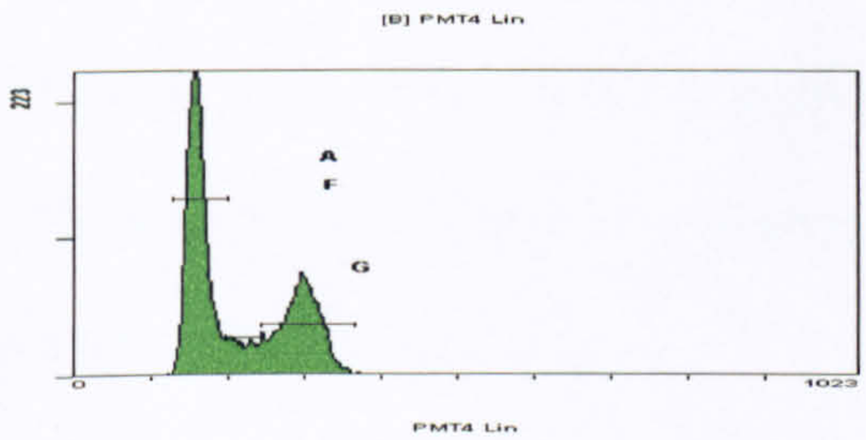


Figure 6.4 Cirsiliol induces G_2/M block in MDA-MB 468 cells. i) Control 0.1% DMSO; 48 hr exposure G_1 62% S 14% G_2 24% ii) Cirsiliol 10 μM 48 hr exposure G_1 42.4% S 18.6% G_2 39%. Experiments were done in duplicate.

i)



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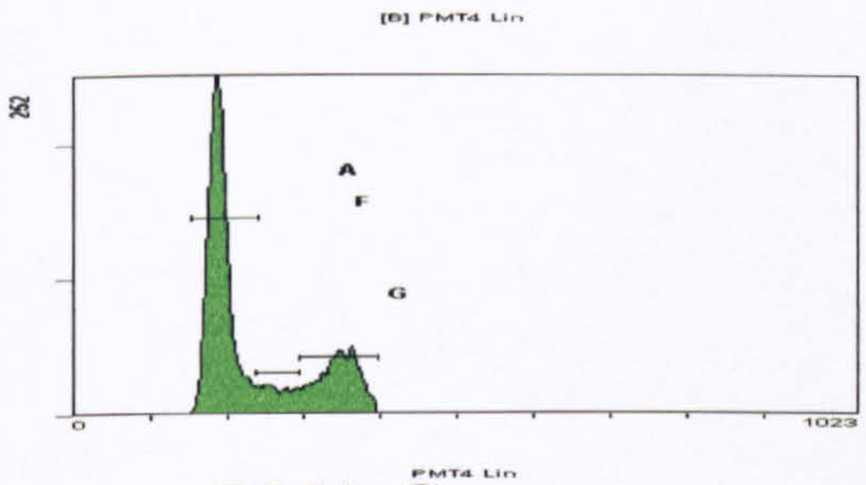
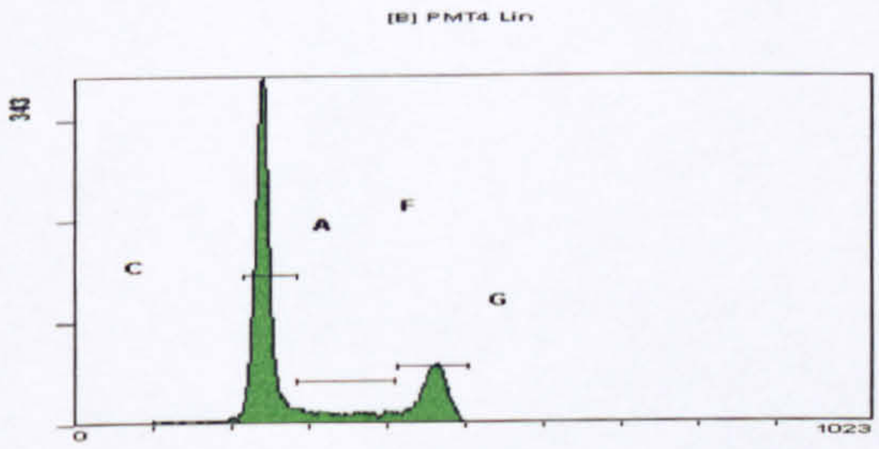


Counts

DNA Content

Figure 6.5 Sinensetin causes growth arrest in the G_1 phase of the cell cycle in MDA-MB 468 cells. i) Control 0.1% DMSO; 30 hr exposure G_1 57% S 14% G_2 29% ii) Sinensetin 10 µM 30 hr exposure G_1 65% S 10% G_2 25%. Experiments were done in duplicate.

ii)



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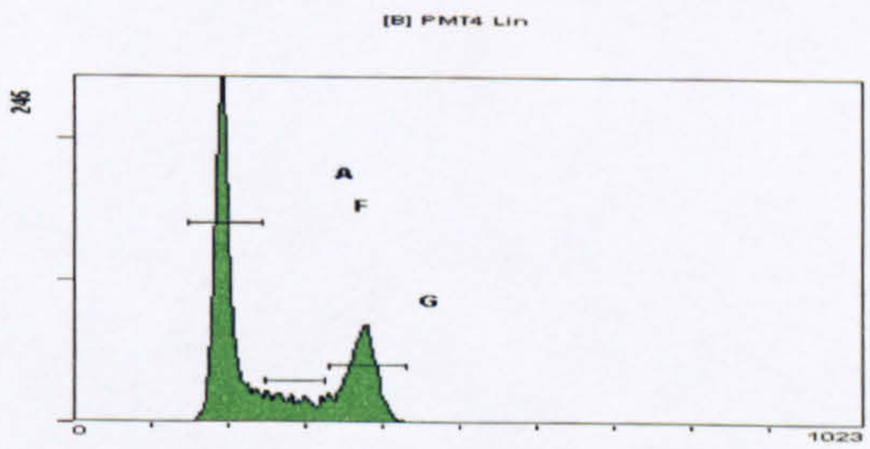


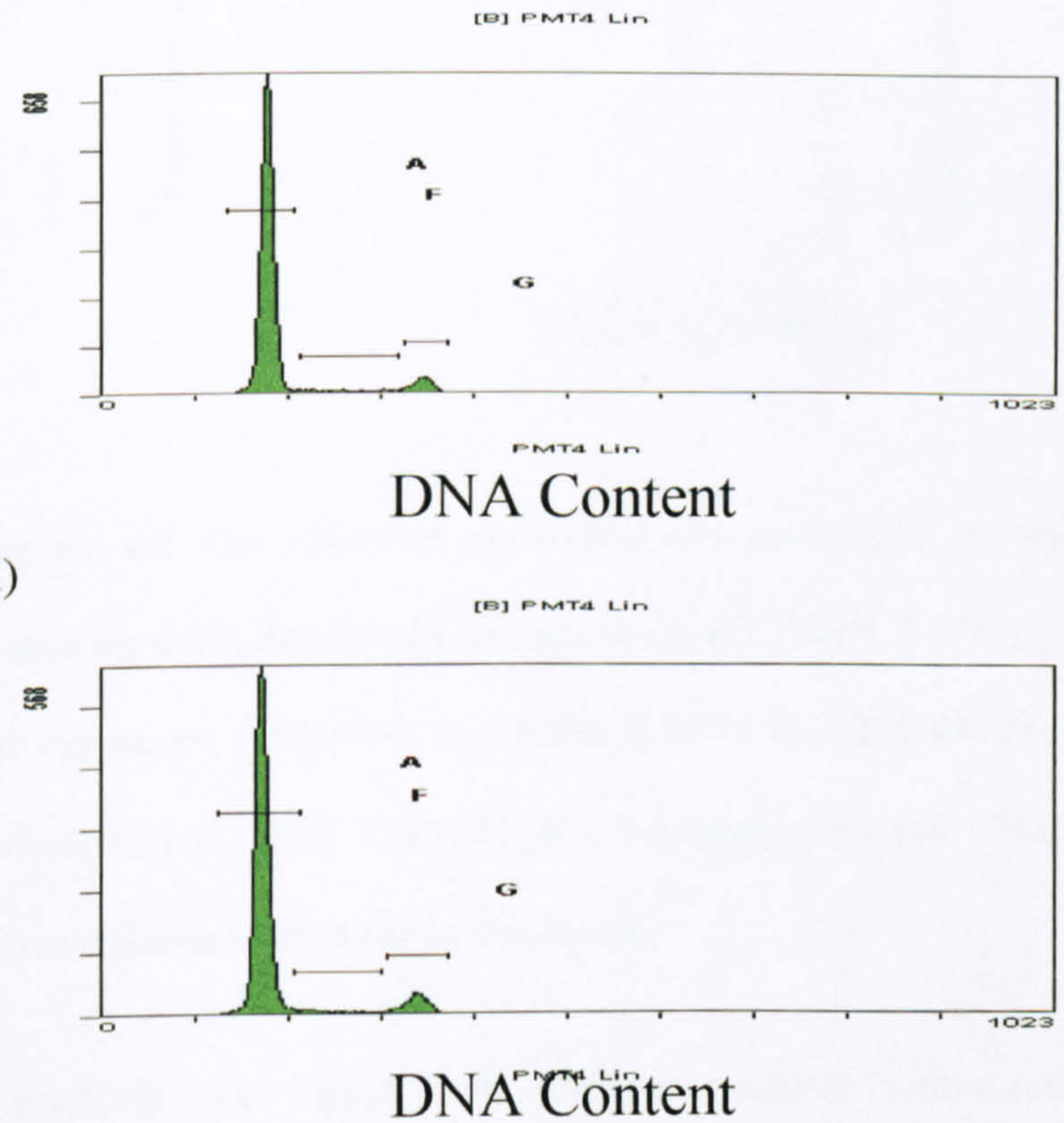
Counts

DNA Content

Figure 6.6 Diosmetin causes growth arrest in the G_1 phase of the cell cycle in MDA-MB 468 cells. i) Control 0.1% DMSO; 48 hr exposure G_1 58% S 14% G_2 28% ii) Diosmetin 10 µM 48 hr exposure G_1 65% S 12% G_2 23%. Experiments were done in duplicate.

In MCF10A cells diosmetin was non toxic at the 48 hr period and did not cause a block in the cell cycle (Figure 6.7). Similar results were obtained for eupatorin-5-methyl ether at 30 hr (data not shown). Eupatorin (10 μM) forced a small fraction of cells to undergo apoptosis at the 30 and 48 hr time period, as shown by the the subG_1 population (5% and 6% respectively) (Figure 6.8). Sinensetin caused a small G_2/M arrest in the 30 hr period in this cell line (Figure 6.8).

i)



ii)

Figure 6.7 Diosmetin is non toxic to MCF10A cells. i) Control 0.1% DMSO; 48 hr exposure G_1 83% S 8% G_2 9% ii) Diosmetin 10 μM 48 hr exposure G_1 83% S 7% G_2 10%. Experiments were done in duplicate.

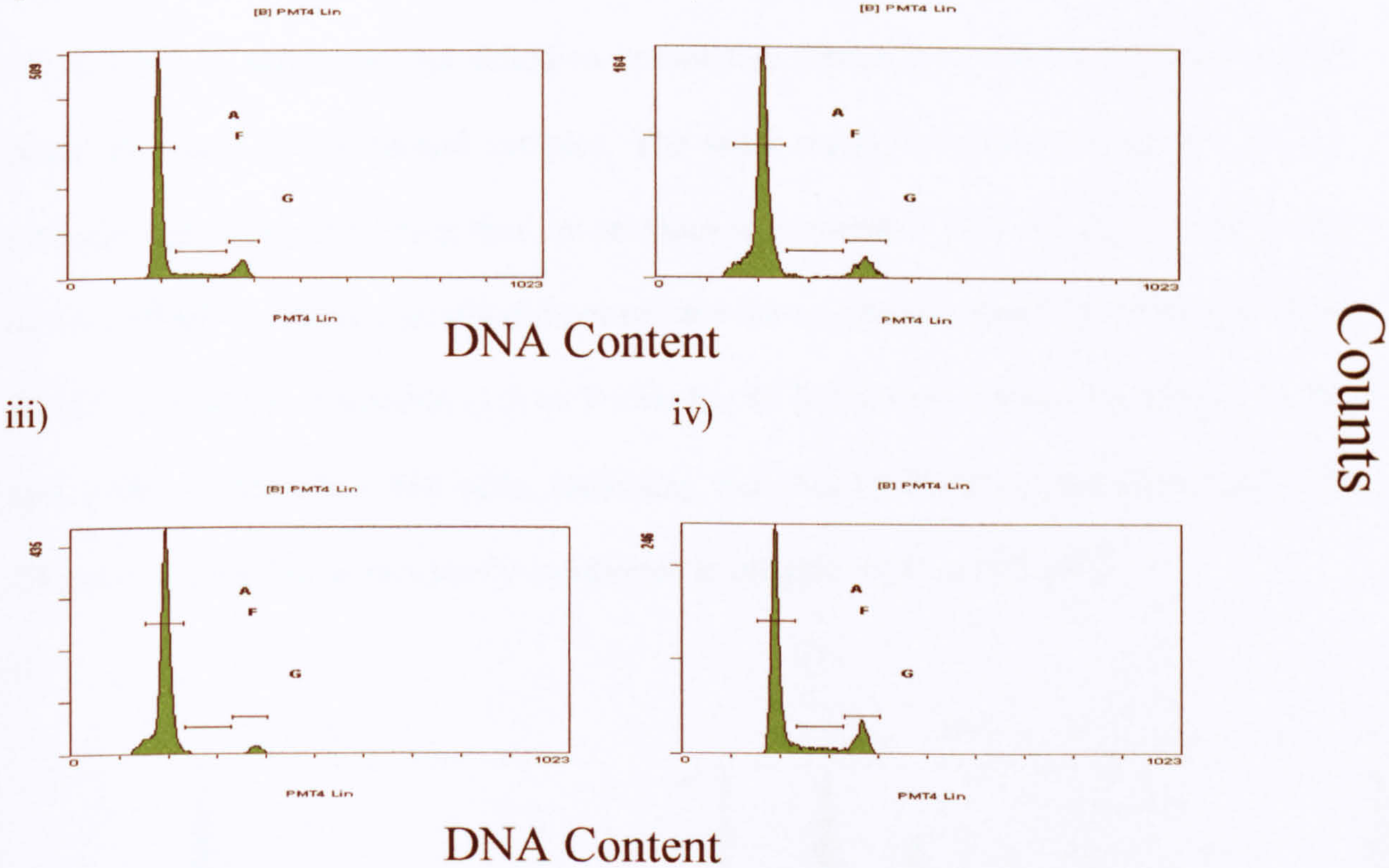


Figure 6.8 The effect of eupatorin and sinensetin on the cell cycle of MCF10A cells. i) Control; 0.1% DMSO 48 hr exposure; G_1 78.3% S 9% G_2 12.7% ii) Eupatorin (10 µM) 30 hr exposure; subG_1 5% G_1 78.3% S 4.7% G_2 12% iii) Eupatorin (10 µM) 48 hr exposure ; subG_1 6% G_1 85% S 3% G_2 6% iv) Sinensetin (10 µM) 30 hr; G_1 71% S 12% G_2 17%. Experiments were done in duplicate.

Eupatorin was found to be the most active compound from all the natural products screened in this type of assay in the MDA-MB 468 cell line, which constitutively express CYP1 family enzymes. Therefore in an attempt to identify whether the cell cycle arrest seen was truly due to metabolism of the flavone from CYP1 enzymes, we focused our

investigation on this compound. Cotreatment of eupatorin (10 μM) and the CYP1 inhibitor acacetin (1.5 μM) resulted in reversal of the G_2/M block, seen in the 48 hr period in the breast cancer cells (Figure 6.9). Acacetin had a significant effect in blocking the activity of eupatorin, but failed to completely return the cells to the initial G_2/M population seen in the control samples. The same result was observed when 3 μM of acacetin were used, indicating that the previous concentration was enough to achieve the desired effect. However acacetin did not reverse the apoptosis noticed in the 30 and 48 hr period. Treatment of acacetin (1.5 or 3 μM) for 48 hrs did not cause any changes in the cell cycle of MDA-MB 468 cells, indicating that this inhibitor did not have any toxic effects (Figure 6.10) as previously mentioned in chapter 5 (IC_{50}=100 μM).

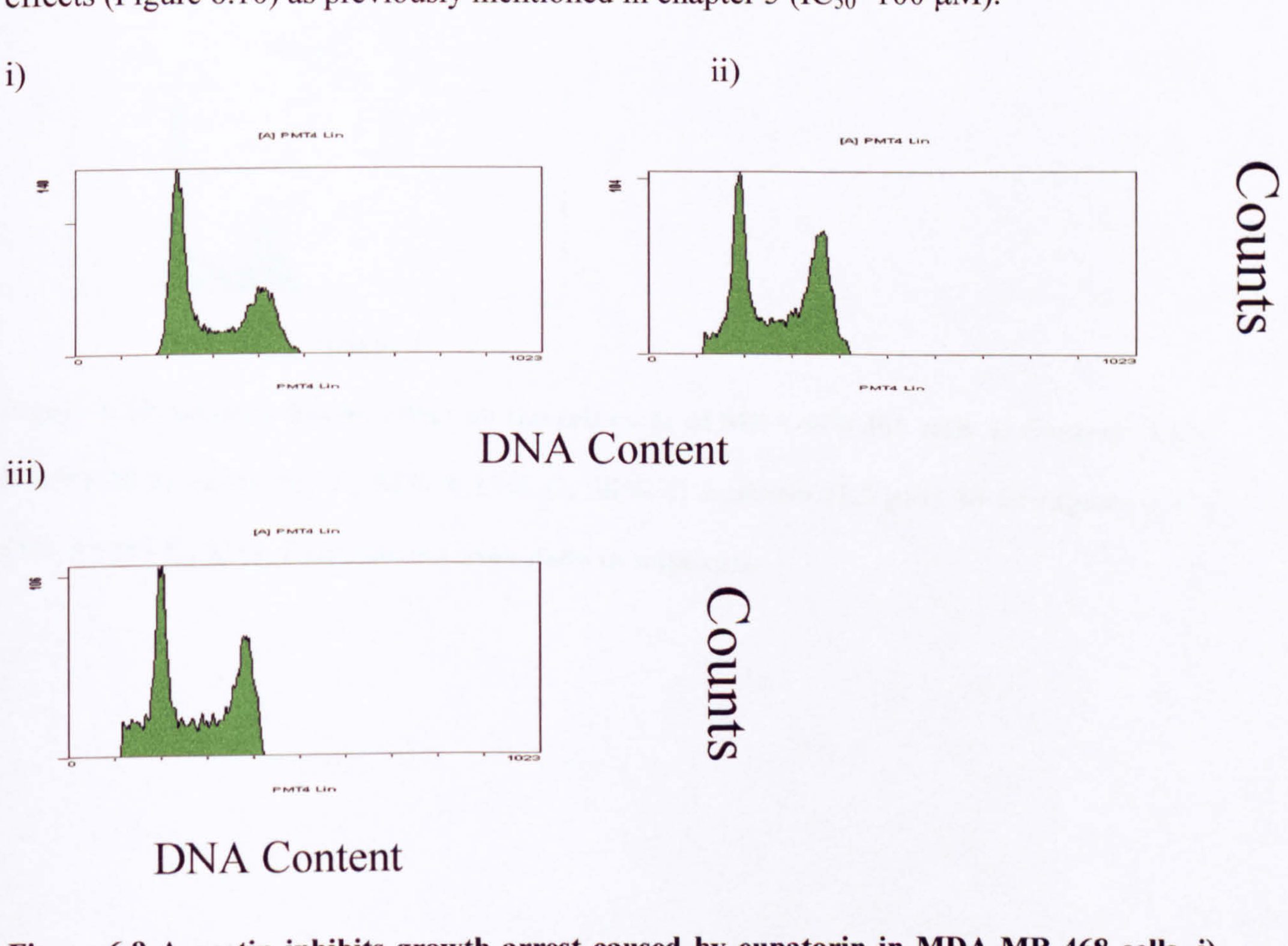


Figure 6.9 Acacetin inhibits growth arrest caused by eupatorin in MDA-MB 468 cells. i) Control; 0.1% DMSO 48 hr exposure; G_1 57% S 14% G_2 29% ii) Eupatorin (10 μM) and

acacetin (1.5 µM) 48 hr exposure; subG$_1$ 3% G$_1$ 43% S 14% G$_2$ 40% iii) Eupatorin (10 µM) and acacetin (3 µM) 48 hr exposure ; subG$_1$ 5% G$_1$ 43% S 15% G$_2$ 37%. Experiments were done in duplicate.

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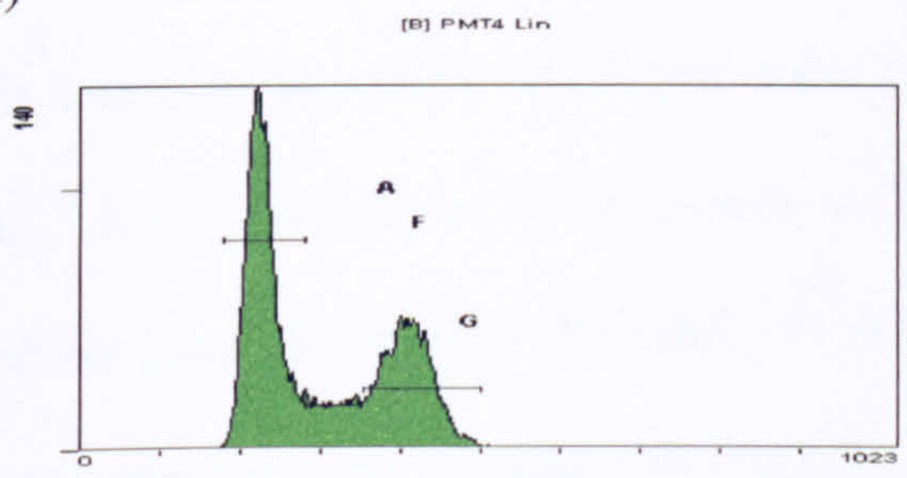

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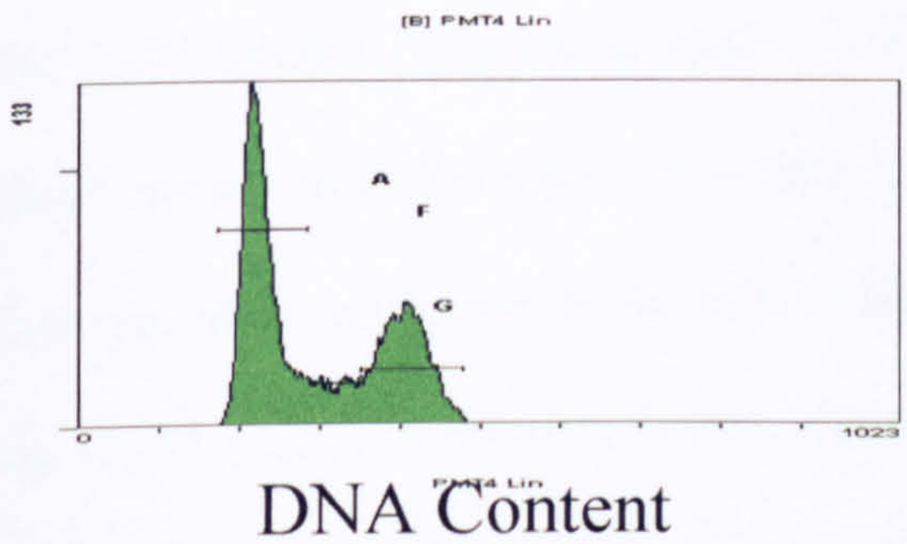


Figure 6.10 Acacetin has no effect on the cell cycle of MDA-MB 468 cells. i) Control; 0.1% DMSO 48 hr exposure; G$_1$ 55% S 15% G$_2$ 30% ii) Acacetin (1.5 µM) 48 hr exposure; G$_1$ 55% S 12% G$_2$ 33%. Experiments were done in duplicate.

In this chapter the inhibitory effects of some of the flavonoids on the cell cycle progression of MDA-MB 468 MCF10A and MCF7 cells was summarised. MCF7 (induced with 10 nM TCDD and non induced) cells did not seem to be affected to notably from treatment with dietary flavones(eupatorin and eupatorin-5-methyl ether). In chapter 5 it was also shown by MTT that these two flavones had the same IC_{50} in both MCF7 and MCF7 preinduced with dioxin cells and this effect was attributed probably to transient expression of CYP1 family enzymes.

Low concentrations of eupatorin (10 μM) seemed to show stronger activity in MDA-MB 468 as opposed to MCF7 cells. This is probably due to constitutive expression of CYP1 enzymes, in this cell line, which is in concordance with previous MTT results (chapter 5). The G_2/M arrest which was caused by eupatorin was reversed by the addition of acacetin, which means that this effect was due to metabolism of eupatorin to an active species. This was also confirmed by the cell cycle analysis in MCF10A cells which did not show any G_2/M block. Previously it was shown, that eupatorin is metabolised to cirsiliol and another two metabolites in the enzyme assay system. In the cell based assay cirsiliol and one of the other two metabolites were detected. Treatment of cirsiliol (10 μM) in MDA-MB 468 cells for 48 hrs showed only a small G_2/M block. This implies that the profound effect which was noticed in the 48 hr period from eupatorin was probably mainly due to the second metabolite rather than to cirsiliol. In the MDA-MB 468 cell line cirsiliol is likely to be metabolised to different products as shown by the IC_{50} difference between the latter cell line and MCF10A (see chapter 5). However the concentration of the parent compound is unlikely to be completely depleted in that time period. Therefore if cirsiliol would cause the profound G_2/M arrest seen in the case of eupatorin incubation with

MDA-MB 468 cells, this effect would have also been observed when cirsiliol was incubated with the latter cells alone. As discussed in chapter 4 and 5 the second metabolite of eupatorin is most probably 6-hydroxyeupatorin or a similar metabolite which contains a hydroxy substitution on the A ring. This metabolite seems to be more active than cirsiliol and probably responsible for the cell cycle arrest noticed in MDA-MB 468 cells.

Induction of apoptosis was evident in MCF10A cells after 30 and 48 hr treatment of 10 μM eupatorin and in the breast cancer cells (MDA-MB 468) with eupatorin (10 μM) and acacetin (1.5 μM) and with eupatorin (10 μM) alone in the same time periods. Hence apoptosis was an intrinscic effect of eupatorin itself and did not occur through metabolic activation.

Eupatorin-5-methyl ether did not have major effect on the cell cycle of MDA-MB 468 cells, suggesting that it might need further incubation time for the metabolism to achieve the desired effect. Sinensetin caused cell cycle arrest in both MCF10A and MDA-MB 468 cells, but from the data obtained it cannot be deduced, whether the result in the breast cancer cells is due to bioactivation of the compound from the CYP1 family enzymes expressed, or an intrinsic effect of the compound itself. Diosmetin had no effect in MCF10A cells and caused a G_1 arrest in MDA-MB 468 cells. Diosmetin is metabolised to luteolin and a second metabolite (D2 or 6-hydroxydiosmetin) in this cell line, while in MCF10A cells there was no metabolsim of this compound as shown in chapter 5. Thus it could be speculated that the effect of diosmetin on the cell cycle of MDA-MB 468 cells is due to metabolism from CYP1 family enzymes.

In the past 15 years particular attention has been given to natural diet based agents capable of inducing selective elimination of cancer cells by inhibiting cell cycle progression and/or causing apoptosis. A lot of studies have investigated the effects of different classes of flavonoids on tumour cells and tried to underpin a link between diet and chemoprevention. The most commonly encountered flavonoids in the literature are hydroxylated flavones or flavonols, such as apigenin, quercetin, kaempferol and luteolin, whereas little is known about the biological activity of polymethoxylated flavonoids such as the previously mentioned eupatorin.

Studies in the early 90s on quercetin showed that it downregulates signal transduction pathways by decreasing PI kinase activity and IP_3 concentration (Singhal et al., 1995). However the concentrations of the compound used for this effect were as high as 60 μM. Early studies on apigenin showed that it induced G_2/M arrest and morphological differentiation, on rat neuronal B104 cells (Sato et al., 1994) but the concentrations used were also very high in this study (30, 60 and 120 μM). Another study showed that apigenin arrests the progression of CA-HPV-10 prostate cancer cells in the G_2 phase of the cell cycle at 5 and 10 μM (Gupta et al., 2001). The same study also showed that apigenin induces apoptosis in the above cell line as evidenced by DNA ladders, fluoresence microscopy and flow cytometry. The authors also investigated the effects of apigenin at 10 μM in the prostate cancer cell lines DU145, LNCap, 22Rv1 and PC-3 and found that it induced apoptosis, which was shown by DNA fragmentation. A similar study examined the effect of apigenin and three other flavonols quercetin, myricetin and kaempferol on HL-60 leukaemic cells (Wang et al., 1999). Apigenin induced apoptosis through a mechanism involving caspase-3 activation (Wang et al., 1999). The other three

flavonols examined had similar effects but the potency followed the order apigenin> quercetin > myricetin > kaempferol. Again these effects were evident at very high concentrations (60 μM).

Agullo and coworkers, examined the inhibitory effects of chrysin, apigenin, luteolin, diosmetin, galangin, kaempferol, fisetin, quercetin, morin and myricetin on PI-3 kinase activity and found that myricetin was the most potent PI-3 kinase inhibitor (Agullo et al., 1997). Structure activity relationships from this study showed that hydroxyl groups on the B ring increase the inhibitory potency of the flavonoids and unsaturation of C2-C3 bond is a necessary requirement for this type of activity (Agullo et al., 1997). PI-3 kinase is a key enzyme involved in signal transduction and cell transformation and therefore there is great interest to develop PI-3 kinase inhibitors, that could be used for the treatment of proliferative diseases. In previous chapters it was shown, that natural flavones with methoxy substituents can get demethylated by CYP1 family enzymes. Hence it can be speculated that this bioactivation process could yield possible PI-3 kinase inhibitors with increased potency, according to aromatic hydroxylation or demethylation reactions taking place.

Flavone has been shown to arrest A549 cells in G_1 phase of the cell cycle at 100 μM through induction of p21 (Bai et al., 1998) and genistein, the isoflavone equivalent of apigenin, induces G_2/M arrest in LNCap prostate cancer cells again through activation of p21 (Kobayashi et al., 2002). Moreover resveratrol has been shown to arrest U937 lymphoma cells in the S phase of the cell cycle at 30-60 μM through a mechanism which is reversed when the compound is removed from the medium (Park et al., 2001).

In the present study it was showen that sinensetin caused G_1 arrest in MDA-MB 468 cells at 10 μM. Tangeretin, a similar flavone, which lacks the 3′-methoxy group that sinensetin has, but contains an extra methoxy group at position 8, has been shown to cause G_1 arrest at 50 μM in COLO 205 cells, through inhibition of CDK4, CDK2 and upregulation of p21 and p53 (Pan et al., 2002). These two compounds are structurally are very similar. With the data presented in this chapter and in chapter 4 it may be speculated that sinensetin exerts its effect probably due to metabolic bioactivation, rather than direct inhibition of cyclin dependent kinases. However sinensetin caused G_2/M arrest in MCF10A cells, which do not express CYP1 enzymes. Unfortunately, there is not enough evidence to demonstrate a possible mechanism of action. Similarly more studies are required to elucidate a mechanism for the G_2/M arrest noticed in the case of eupatorin.

The effect of diosmetin on the cell cycle of MDA-MB 468 cells is in agreement with previous data published in the literature. Diosmetin is metabolised to luteolin (chapter 5) and luteolin has been previously shown to inhibit the autophosphorylation of the EGFR (Huang et al., 1999). G_1 arrest of the cell cycle would be expected since the progression of G_1 to S phase is mainly dependent on mitogenic signals with the transcription of the cyclin D_1 gene occuring via the Ras/Raf/ERK pathway, as mentioned in chapter 1 section 1.1.5.7. Progression through the S and the G_2/M phase is dependent to a smaller extent to mitogenic stimuli. Indeed 12 hr treatment of DU145, PC3 and LNCap prostate cancer cells with the potent EGFR inhibitor Iressa or ZD1839 caused G_1 arrest (Sgambato et al., 2004). In CAL33 cells 24 hr treatment of Iressa also caused a G_1 block (Magne et al., 2003). These supportive evidence provide more insight about the chemopreventative action of diosmetin: Diosmetin causes G_1 arrest in MDA-MB 468 cells, because of

metabolism to luteolin from CYP1 family enzymes. Diosmetin is bioactivated into an EGFR tyrosine kinase inhibitor in this cell line, whereas no effect is observed in the normal breast MCF10A cells, which do not express CYP1 enzymes. MDA-MB 468 cells have been shown to express EGFR, in the absence and presence of serum, and at least one order of magnitude greater than the normal breast cell line HBL100 (Squires et al., 2003).

MDA-MB 468 cells have been used as a model cell line to investigate modulation of key survival pathways by the polyphenolic curcumin in a recent study (Squires et al., 2003). In this study curcumin caused G_2/M arrest at 20 μM, following 48 hr incubation, which was also evident in a normal cell line (HBL100), through inhibition of the ERK pathway (Squires et al., 2003). Curcumin was also shown to exert the above biological effect through inhibition of c-jun phosphorylation and JNK activity (Squires et al., 2003). Even though, no plausible mechanism of action for eupatorin's growth arrest was shown, it is worthwhile noticing, that it is a lot better chemopreventative candidate than curcumin. Eupatorin caused higher G_2/M arrest in MDA-MB 468 cells (subG_1 5%, G_1 17%, S+G_2 78%) than curcumin (G_1 27%, S+G_2 73%) at a lower concentration (10 instead of 20 μM) and in parallel it had little toxicity in normal breast MCF10A cells. This is due to selective bioactivation occurring in the cancer cells and not in the normal cells, from CYP1 family enzymes as discussed earlier. Curcumin probably does not get metabolised in the same cell line, since the structure of the molecule is too big to fit the active sites of the enzymes and therefore it acquires its biological effects in both normal and cancer cells through different mechanisms of action.

Even though the studies in the literature discussed earlier, propose that flavonoids inhibit cell growth through inhibitory effects on the major signalling pathways and cyclin dependent kinases, the concentrations used in most of these studies were artificially higher (up to 100 times higher) than the physiological levels of the flavonoids in human blood serum. The results presented here, show that flavones such as eupatorin could be very promising chemopreventative molecules, which can cause selective cell cycle arrest in cancer cells at concentrations closer to physiological levels, through metabolic activation from CYP1 family enzymes.

7. THE INDUCTION OF CYP1 ENZYME EXPRESSION FROM DIETARY FLAVONOIDS IN TUMOUR CELLS

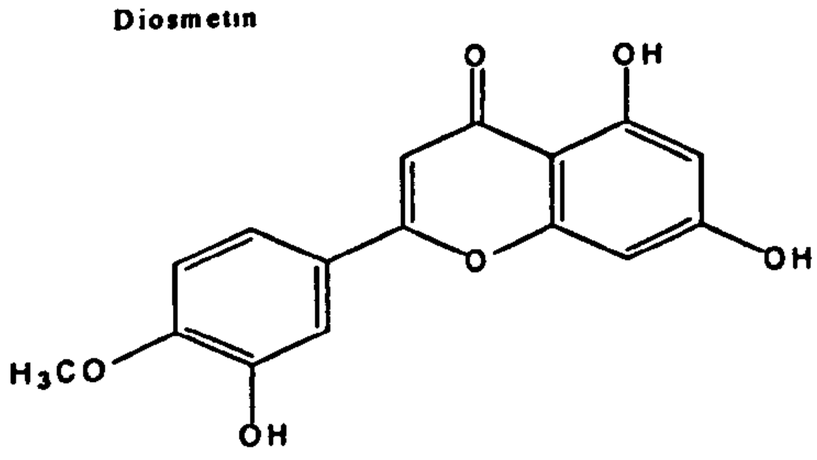
7.1 Introduction

As discussed in chapter 1 PAHs can be metabolised to carcinogens by the CYP1 family of enzymes mainly, CYP1B1 and CYP1A1. The mechanism of their action requires induction of these enzymes via the aryl hydrocarbon receptor (AhR). The best characterised molecular response to ligands of the AhR is the induction of transcription of *CYP1A1*. A number of chemical inducers of CYP1A1 and CYP1B1 has been identified e.g 3-methylcholanthracene, Benzo[*a*]pyrene, TCDD and dimethylbenz[*a*]anthracene(Kim, 1998, Shimada, 1996). Surprisingly several natural products have also been shown to act as agonists or antagonists of the AhR and to induce *CYP1* expression. The most widely studied natural "dioxin" or AhR agonist is indole-3-carbinol (I3C) (Fong et al., 1990). This compound is a major secondary metabolite found in cruciferous vegetables (i.e. cabbages, Brussels, sprouts, broccoli), which induces phase I and phase II drug metabolising enzymes in laboratory animals (Kojima et al., 1994) I3C also inhibits several carcinogen induced tumours at various sites in rodent models and decreases the development of spontaneous mammary and endometrial tumors in female mice (Chen et al., 1996). I3C is a weak agonist of AhR in T47D cells (human breast cancer cells), as far as *CYP1A1* mRNA levels are concerned, but is shown to significantly inhibit TCDD induced EROD activity in this cell line (Chen et al., 1996). However the concentrations used in this study were significantly higher than the possible levels of the compound circulating in plasma.

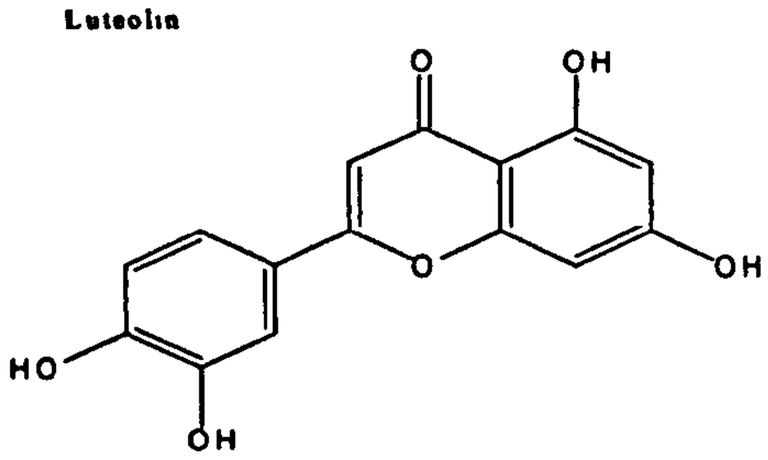
The hypothesis, whether the flavones that have been shown in the previous chapters to be substrates for the CYP1 family enzymes could act in a similar way as I3C and induce the expression of phase I metabolising enzymes, was examined. The research focused on the

CYP1 family enzyme expression in breast cancer cells. MCF7 cells were used as a model for these experiments. The structures of the compounds investigated in this type of studies are shown in Figure 7.1

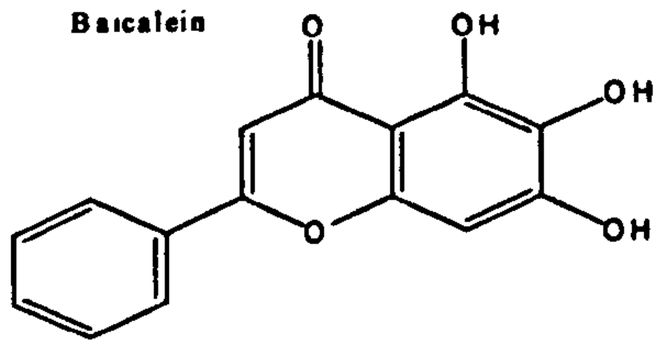
Diosmetin



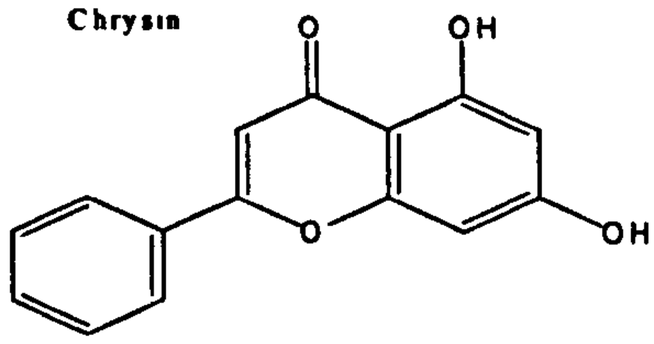
Luteolin



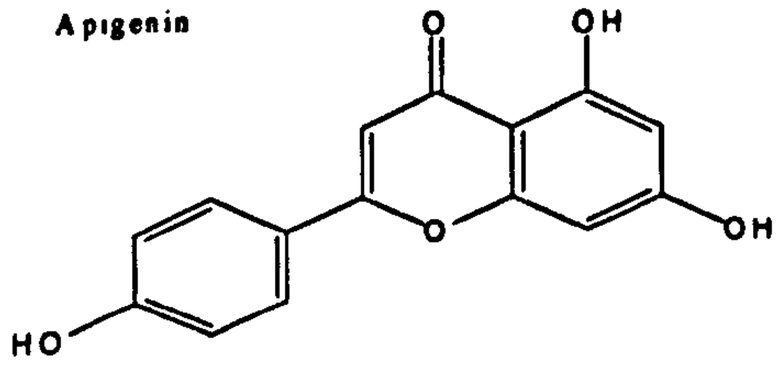
Baicalein



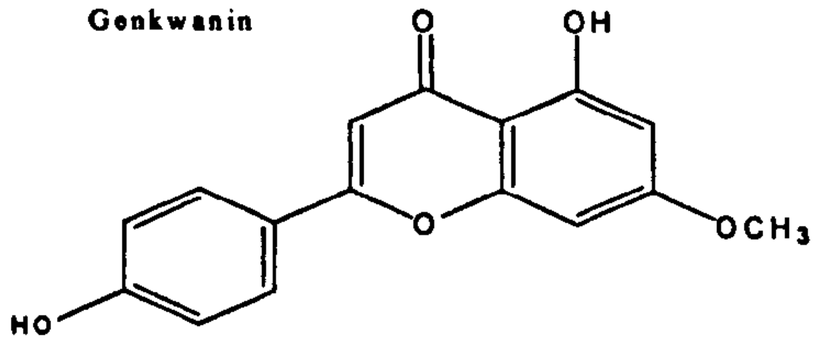
Chrysin



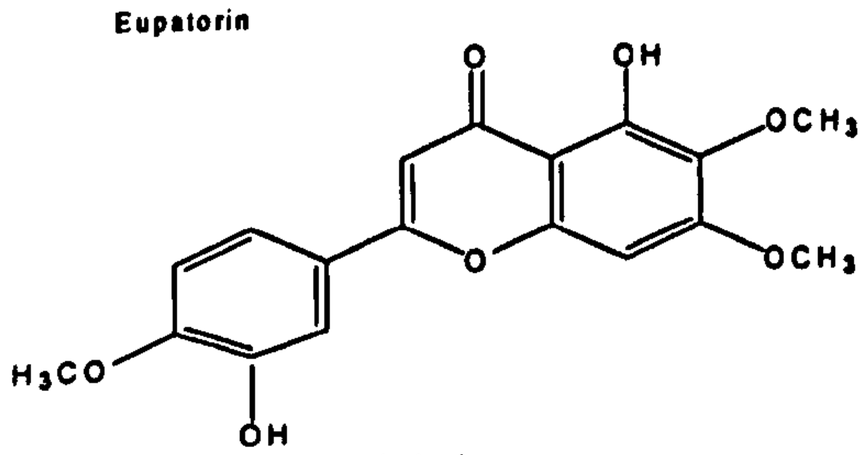
Apigenin



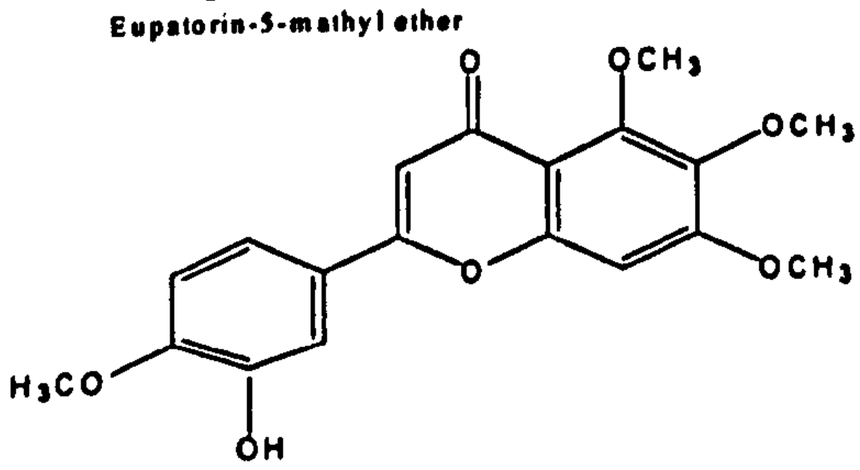
Genkwanin



Eupatorin



Eupatorin-5-methyl ether



Cirsiliol

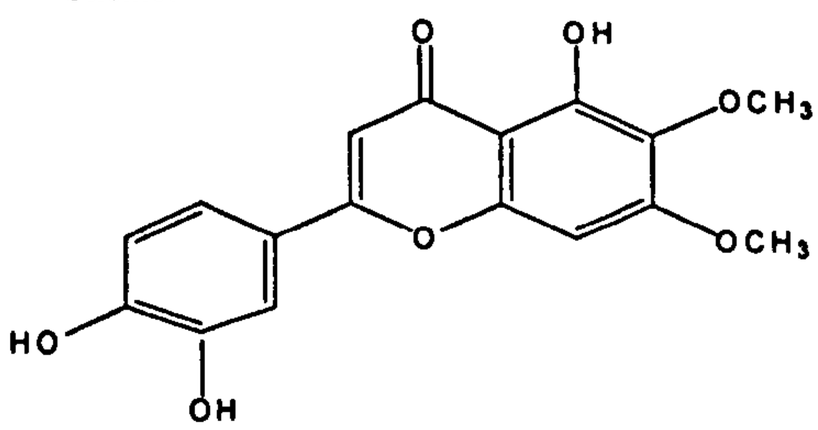


Figure 7.1 Structures of the flavonoids investigated in the induction assays

7.2 Results and discussion

7.2.1 Comparison of the two RNA extraction methods

The difficulties associated with the isolation of total RNA are usually due to ribonuclease (RNase) activity. RNases are a family of enzymes that degrade RNA through both endonucleolytic and exonucleolytic activity. Before proceeding into any other step of experimental work, it is essential to rule out any contamination possibility. In general, running the RNA on a denaturing gel is the single best diagnostic test. Formation of the 28S and 18S ribosomal RNA (rRNA) on the gel is a good sign that the RNA is intact. One should observe a minimum of smearing above between and below these major RNA species. Lack of definition to the rRNA usually means that the sample has been subjected to nuclease degradation. The intensity of the 28S rRNA is about twice of that of 18S. An intact RNA sample manifests its mRNA component as a significantly lighter smearing above, below and between the rRNAs.

Both the acid-phenol RNA extraction method and the column-based RNA extraction method yielded RNA of satisfactory quality as shown by Figure 7.2. However the column method was selected due its speed and simplicity, making RNA extraction possible within just two hours. The acid-phenol extraction requires the preparation of a number of solutions, which also increases the risk of the RNA being more susceptible to degradation by Rnases, each time the extraction is carried out.

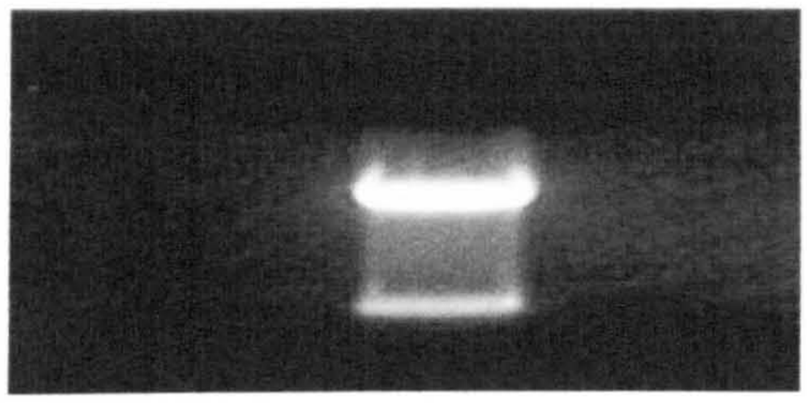


Figure 7.2 RNA gels of samples isolated from MCF7 cells from the acid-phenol (bottom) and the column method (top). Dark areas indicate the staining from bromophenol blue and xylene cyanol

7.2. 2 Selection of the cell line and optimisation of the assays

The MCF7 cell line was used as a model, to investigate whether dietary flavonoids (Figure 7.1) can induce expression of CYP1 enzymes. MCF7 cells have been used in the past by other studies (Ciolino et al., 1998, Ciolino and Yeh, 1999), for these type of assays and therefore the results generated could be comparable to already published results in the literature. Two other cell lines were tested in our laboratory, but did not show significant expression of CYP1 family enzymes after treatment with TCDD. MDA-MB 231 and UT-14 cells were treated with TCDD (10nM) for 16 hours and the EROD activity is shown in Figure 7.3. TCDD had no effect on the induction of CYP1 enzyme expression. The fluoresence obtained in both control and TCDD samples treated samples

was probably due to background signal from ethoxyresorufin itself. In a previous experiment the cells were treated with TCDD (10 nM) for 24 hours and dietary flavones (concentration range 5-0.625 μM) and similar results were obtained i.e. virtually no increase in EROD activity was observed.

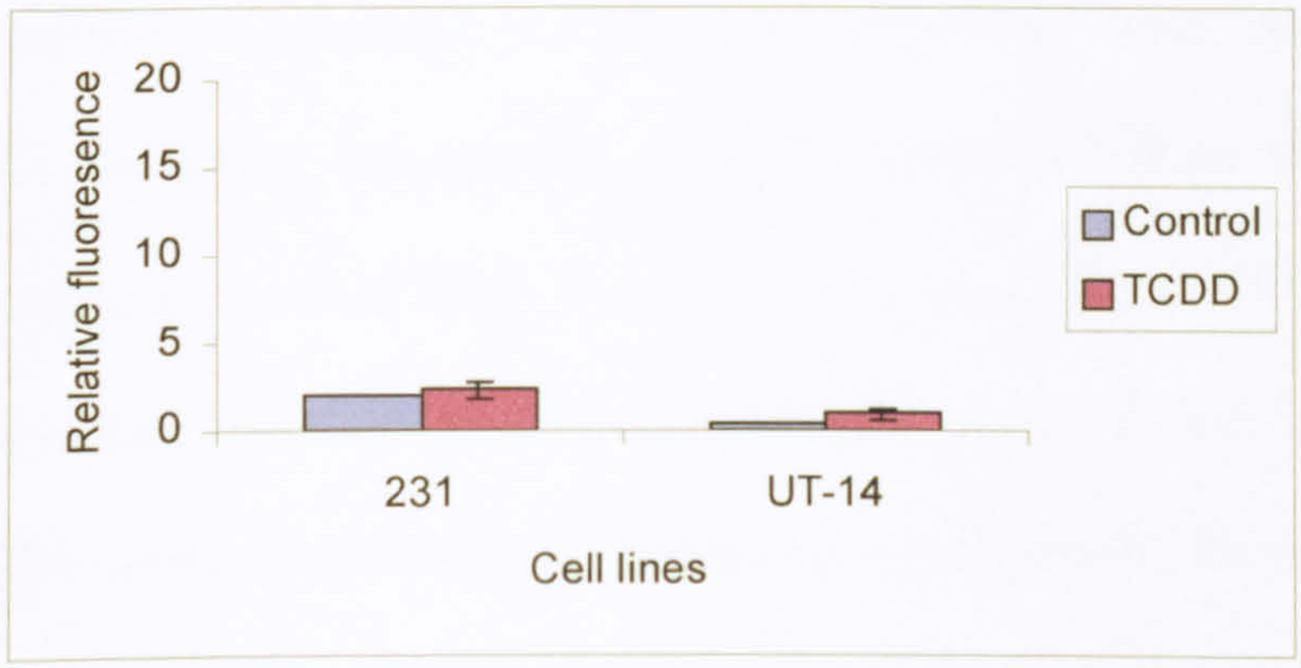


Figure 7.3 EROD activity of MDA-MB 231 cells and UT-14 cells with and without treatment of 10 nM TCDD for 16 hours

EROD assay in MCF7 cells is usually performed for one hour as described above. The fluoresence yielded from the conversion of resorufin was measured over time and as shown in Figure 7.4 50 minutes was chosen, as a time point, to represent the linear portion of the curve. For a primary screen EROD-dealkylation activity was measured as relative fluoresence units. Once a lead compound was identified fluoresence was converted to units of pmol of resorufin/min/number of cells present.

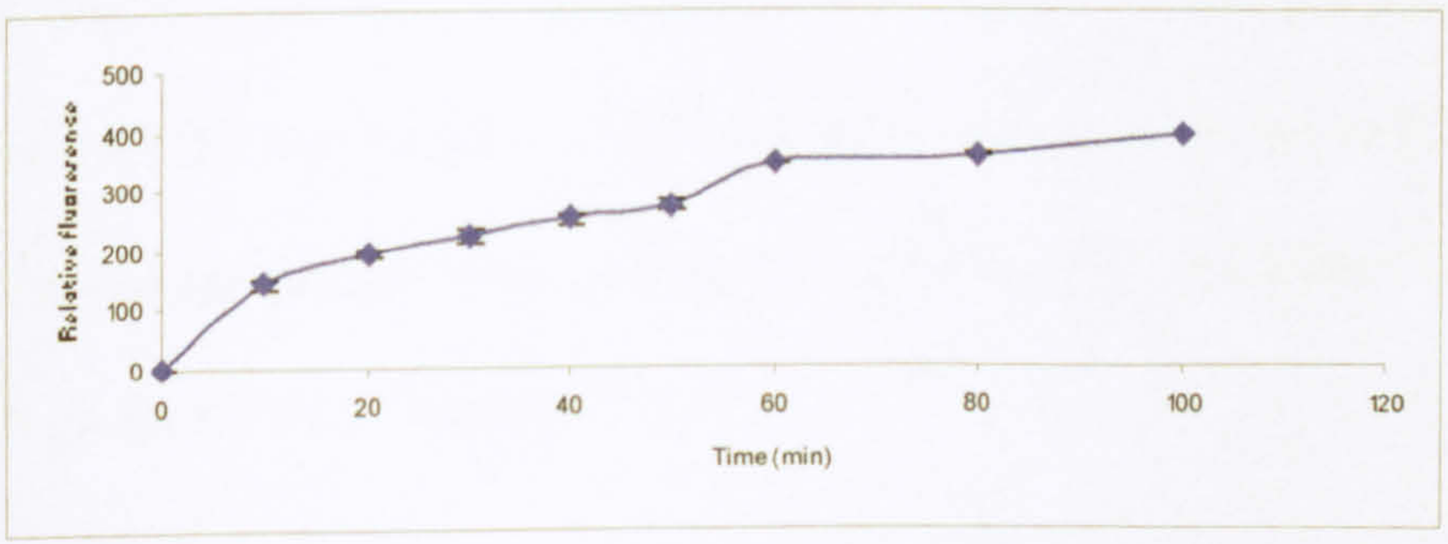


Figure 7.4 Time dependence of resorufin formation

The primers for the PCR amplification of *CYP1A1* and *CYP1B1* cDNA were obtained from Dohr et al., 1995. However their protocol which contained all the necessary parameters, was found to be not reproducible. The critical points in the PCR are the annealing temperature and the primer sequence. Since the primers were already obtained the annealing temperature could be estimated from the primer sequence from the empirical formula: 61.8 °C + [(G+C) x 41- 675] / Σ(A+T+G+C). This estimation would give two temperatures one corresponding to the forward and one to the reverse primer. The optimum annealing temperatures would usually be somewhere close to the average. For *CYP1A1*, *CYP1B1* and *β-actin* the temperatures were calculated to be 51,53,51,53 and 61 and 52 °C for the forward and reverse primer respectively. β-Actin is a housekeeping gene and it is used because its expression throughout the different cell types remains relatively constant (Sambrook and Russel, 2001). 60 °C was the annealing temperature provided from the protocol for both *CYP1A1* and *β-actin* and the cycle numbers 23 and 20 respectively. No band was obtained under these conditions. The temperature was lowered to 55 °C and the results are shown in Figure 7.5. The marker used is a 100 base pair ladder (lane 1 and 2), starting from the lower band and going to up (100, 200, 300 etc.). A faint band was produced for *β-actin* and a very faint band for one of the *CYP1A1* samples. Cycle number was also increased to 25. The very small bands at the end are primer homodimers, indicating that 0.4 μM primer final concentration is quite high for *CYP1A1* fragment.

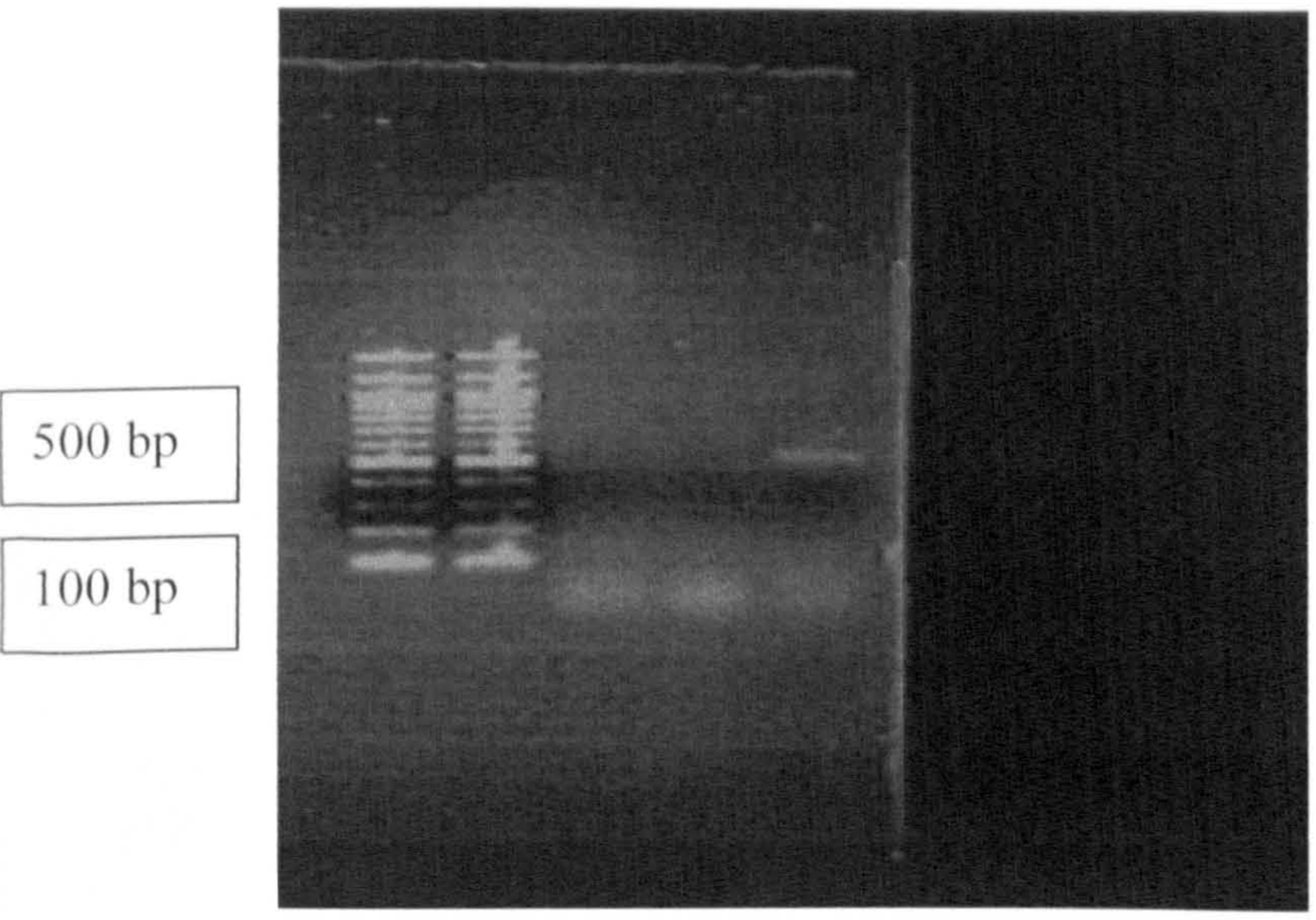


Figure 7.5 RTPCR of RNA samples isolated from MCF 7 cells, which were induced for 24 hours with TCDD. Lane 1 and 2: 100bp ladder, Lane 5: *β-actin*, Lane 3 and 4: *CYP1A1*

The annealing temperature was lowered to 52 °C and the cycle number used was 25. Three samples again were tested and two bands were obtained for *CYP1A1* and no band for *β-actin*, which indicated that the optimum annealing temperature is 52 and 55 °C respectively. 100 bp ladder was used again. The more intense bands in the ladder represent the starting fragment size (100 bp), 500 bp and 1 Kbp DNA fragments.

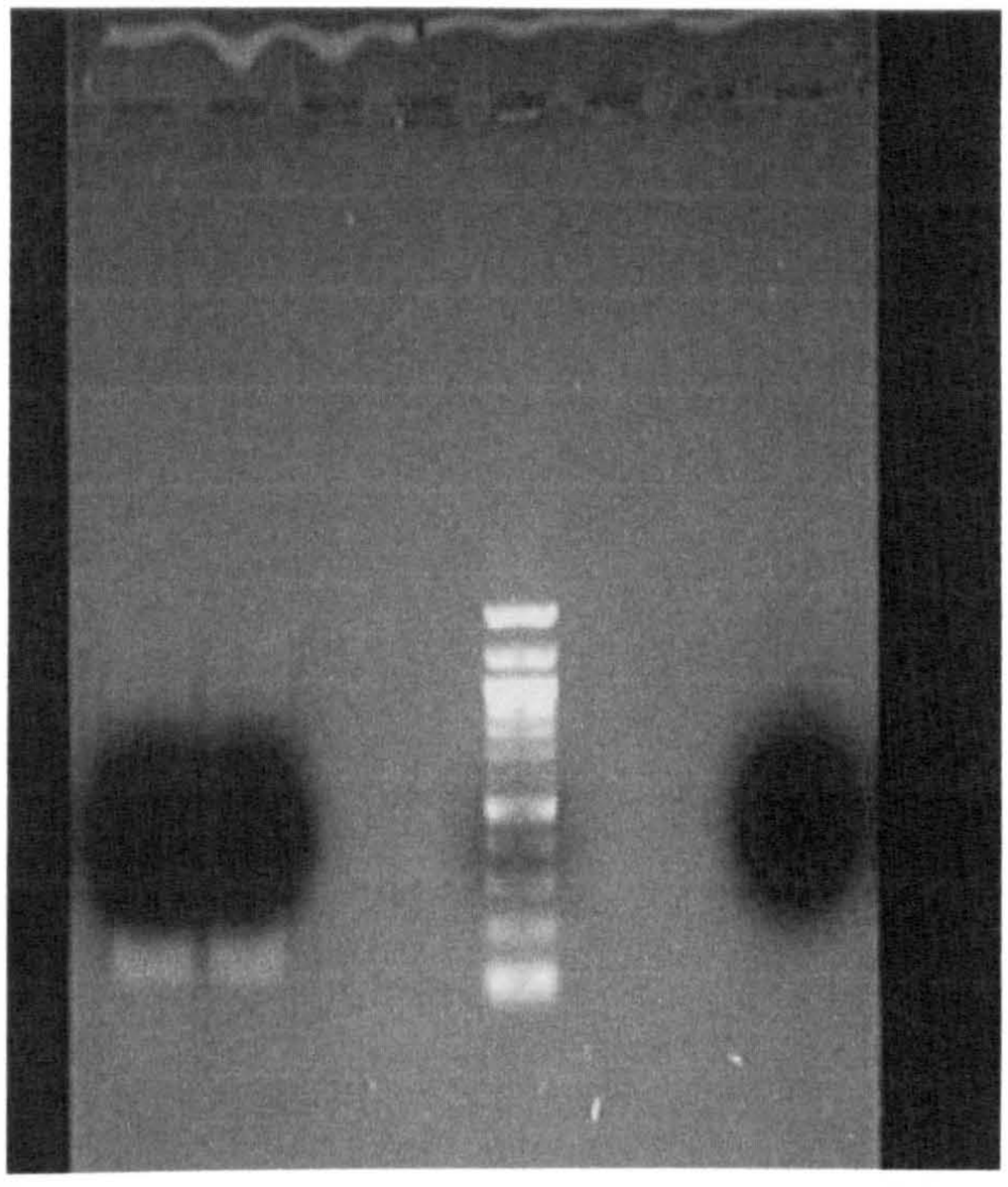


Figure 7.6 RTPCR of RNA samples isolated from MCF 7 cells, which were induced for 24 hours with TCDD. Lane 5: 100bp ladder, Lane 8: *β-actin*, Lane 1 and 2: *CYP1A1*.

Once the annealing temperature and the cycle number were optimised the last parameter to be optimised was cDNA concentration, so that a clear comparison for a concentration and time dependent change in the *CYP1A1* and *CYP1B1* mRNA levels in MCF7 cells could be shown. Figure 7.7 shows an example of the amplification of two cDNA samples with different initial concentration, for amplifying the *β-actin* gene. The final cDNA concentration in the PCR, which would be optimum would be close to 0.08 mg/ml.

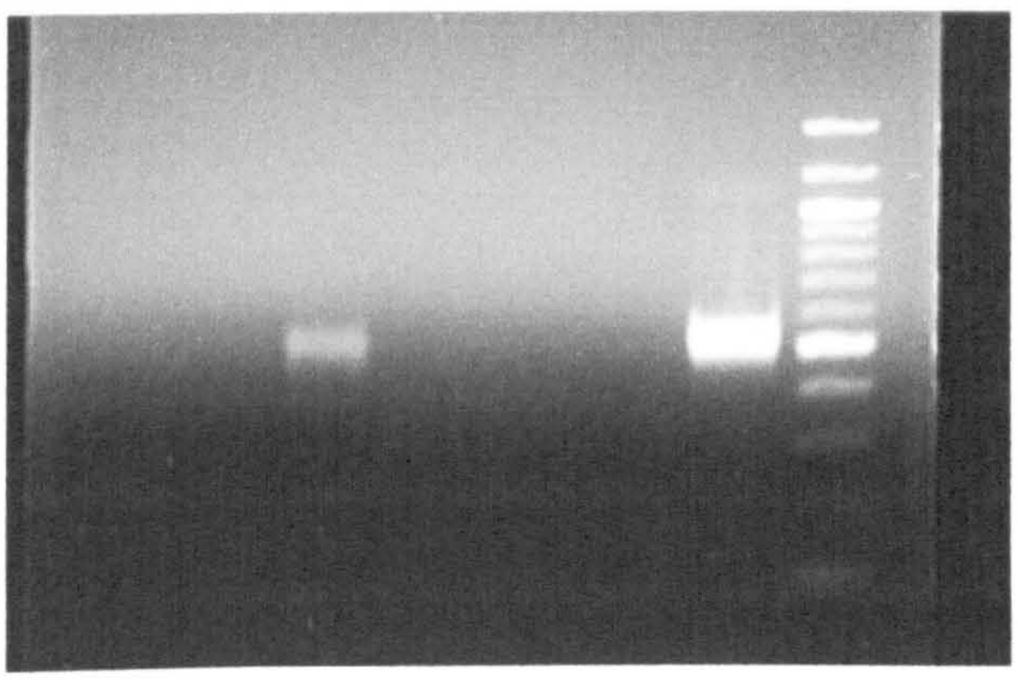


Figure 7.7. RTPCR of RNA samples isolated from MCF 7 cells. Non treated cells *β-actin* (lane 3 0.03 mg/m final cDNA conc and lane 7 0.12 mg/ml final cDNA conc).

Following the results obtained from the previous experiments the annealing temperature for *CYP1B1* was set to be 52 °C from forward and reverse primer. The cycle number used was between the range 25-30. Moreover the expected primer concentration would fall in the range of 0.3-0.4 μM, since the *CYP1B1* fragment was nearly 400 base pairs. A typical example of the DNA fragment corresponding to *CYP1B1* is shown in Figure 7.8. 1 kbp ladder was used. The more intense bands in the ladder represent the starting fragment size (1000 bp), and heavier DNA fragments (2kb, 3kb etc.).

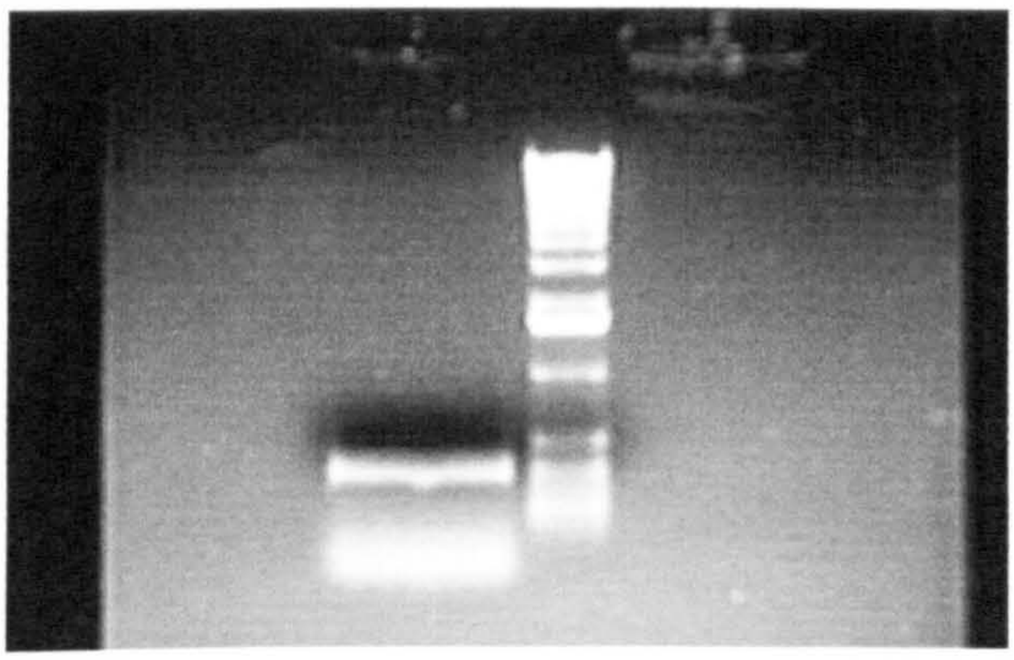


Figure 7.8 RTPCR of RNA sample isolated from MCF7 cells, which was induced for 24 hours with TCDD. Lane 3: *CYP1B1* Lane 4: 1kb ladder

7.2.3 Natural occurring dietary flavones induce CYP1B1 and CYP1A1 expression in MCF7 cells

MCF7 cells were treated with flavones at different concentrations for 24 hours and the amount of CYP1 enzyme activity was measured using the EROD assay. Preliminary experiments had showed that chrysin and baicalein did not induce CYP1 enzyme expression at 1.25 μM (Figure 7.9). Luteolin (Figure 7.9) and diosmetin (Figure 7.10) caused a small increase in CYP1 enzyme activity at the same concentration. Apigenin did not have a major effect at concentrations of 5, 2.5 and 1.25 μM (Figure 7.11). All these compounds were tested in the cytotoxicity assay and the data are presented in chapter 5. Apigenin and luteolin had IC_{50s} of 50 and 40 μM respectively in MCF7 cells (data not shown).

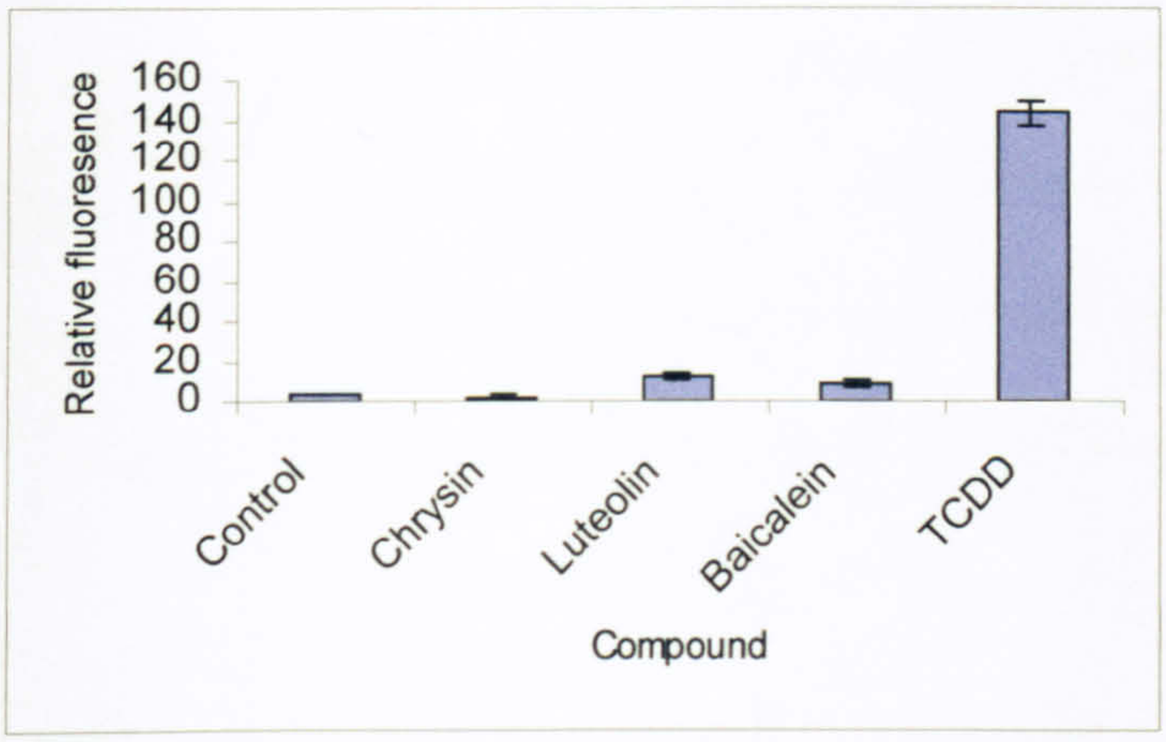


Figure 7.9 EROD activity of MCF7 cells treated with natural products (1.25 μM) for 24 hours. 10nM of TCDD and 0.1% DMSO were used for positive and negative control respectively. Blue bars and error bars represent mean ± standard deviation of three replicates respectively.

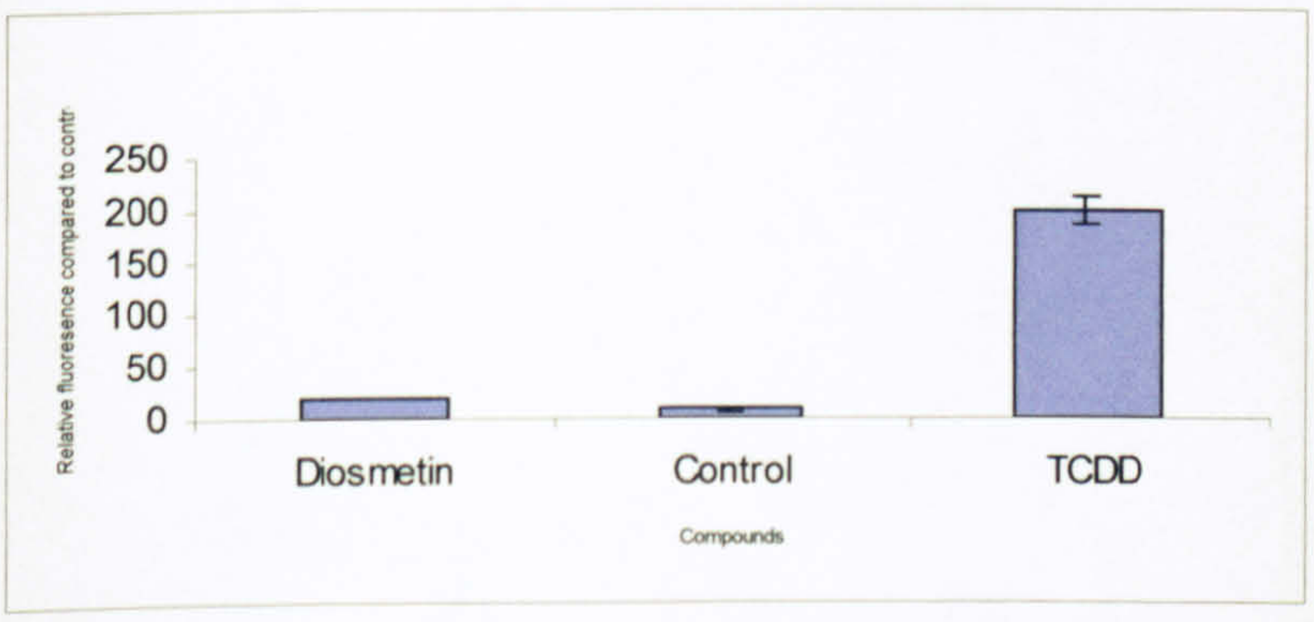
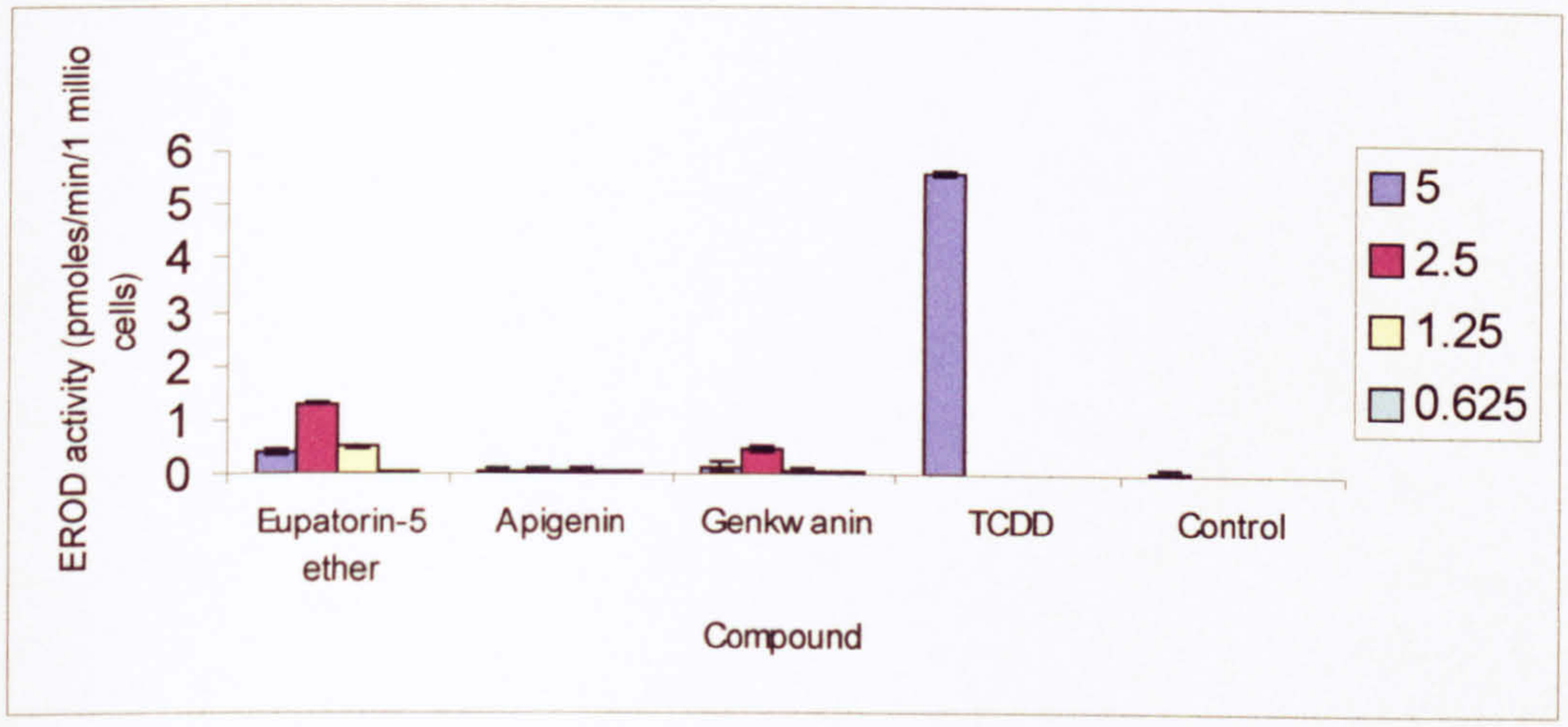


Figure 7.10 EROD activity of MCF7 cells treated with diosmetin (1.25 μM)for 24 hours. 10nM of TCDD and 0.1% DMSO were used for positive and negative control respectively. Blue bars and error bars represent mean ± standard deviation of four replicates respectively.

The flavones eupatorin, eupatorin-5-methyl ether, cirsiliol and genkwanin induced CYP1 enzyme expression after 24 hours in a concentration dependent manner (Figure 7.11). The major induction was noticed at 2.5 μM, whereas at 5 μM the activity started to

decline. The most potent inducer was cirsiliol. Eupatorin and eupatorin-5-methyl ether are weaker inducers of CYP1 enzyme activity. Genkwanin was also a weak inducer. The increase in CYP1 activity in MCF7 cells caused by treatment of natural flavones was compared to TCDD, which was also used for positive control. It is intresting to note that the induction by 2.5 μM cirsiliol was nearly half that of 10 nM TCDD.



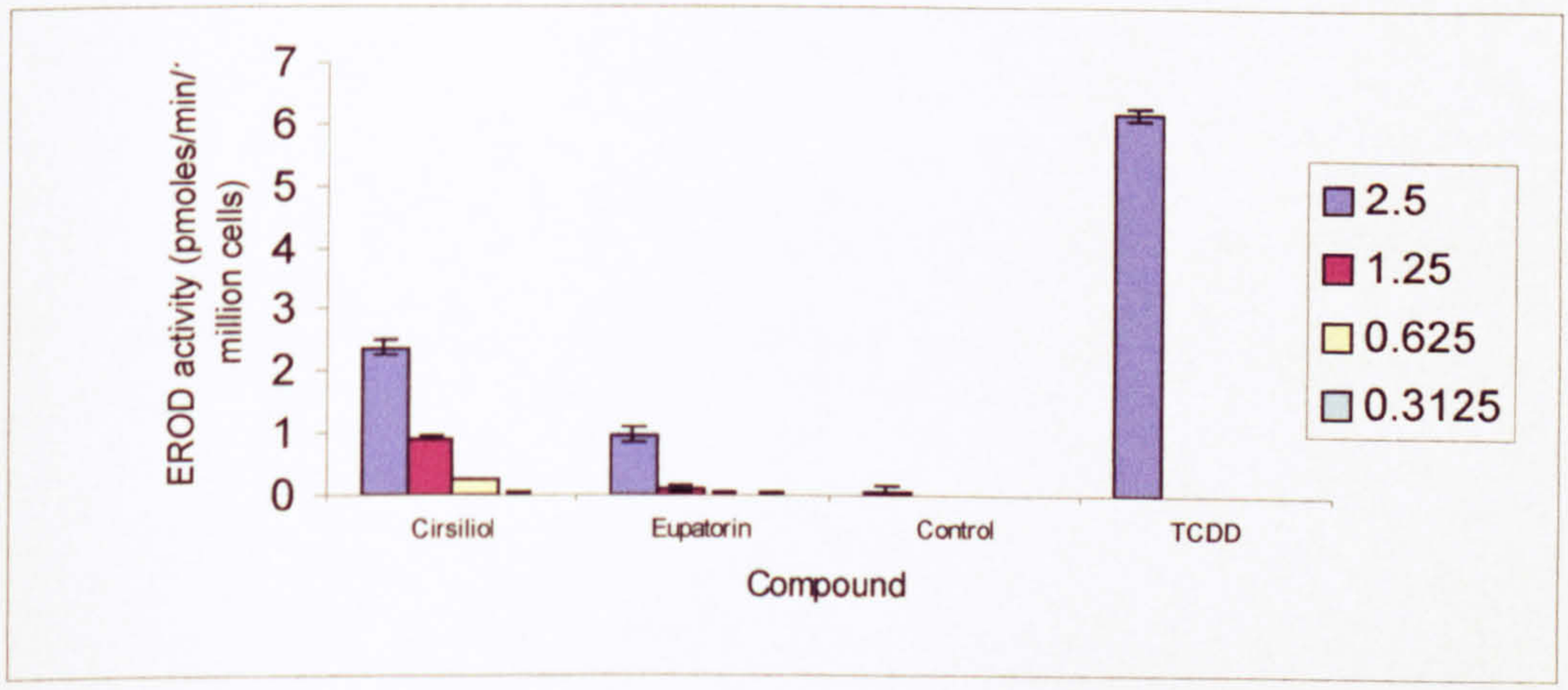


Figure 7.11 The effect of different concentrations of flavonoids on the expression of CYP1 family enzymes in MCF7 cells after 24 hours. TCDD was used at 10 nM as positive control and 0.1% DMSO as negative control. Concentration range included 0.3125, 0.624, 1.25, 2.5 and 0.625, 1.25, 2.5, 5 μM respectively. Experiments were done in triplicate. Error bars represent mean ± SD.

The time dependence of the CYP1 enzyme induction from the flavonoids was also investigated. After 24 hours incubation with 2.5 μM of flavones CYP1 enzyme activity declined (Figure 7.12). The flavonoid-induced increase in CYP1 activity was therefore maximal after a 24 hour period.

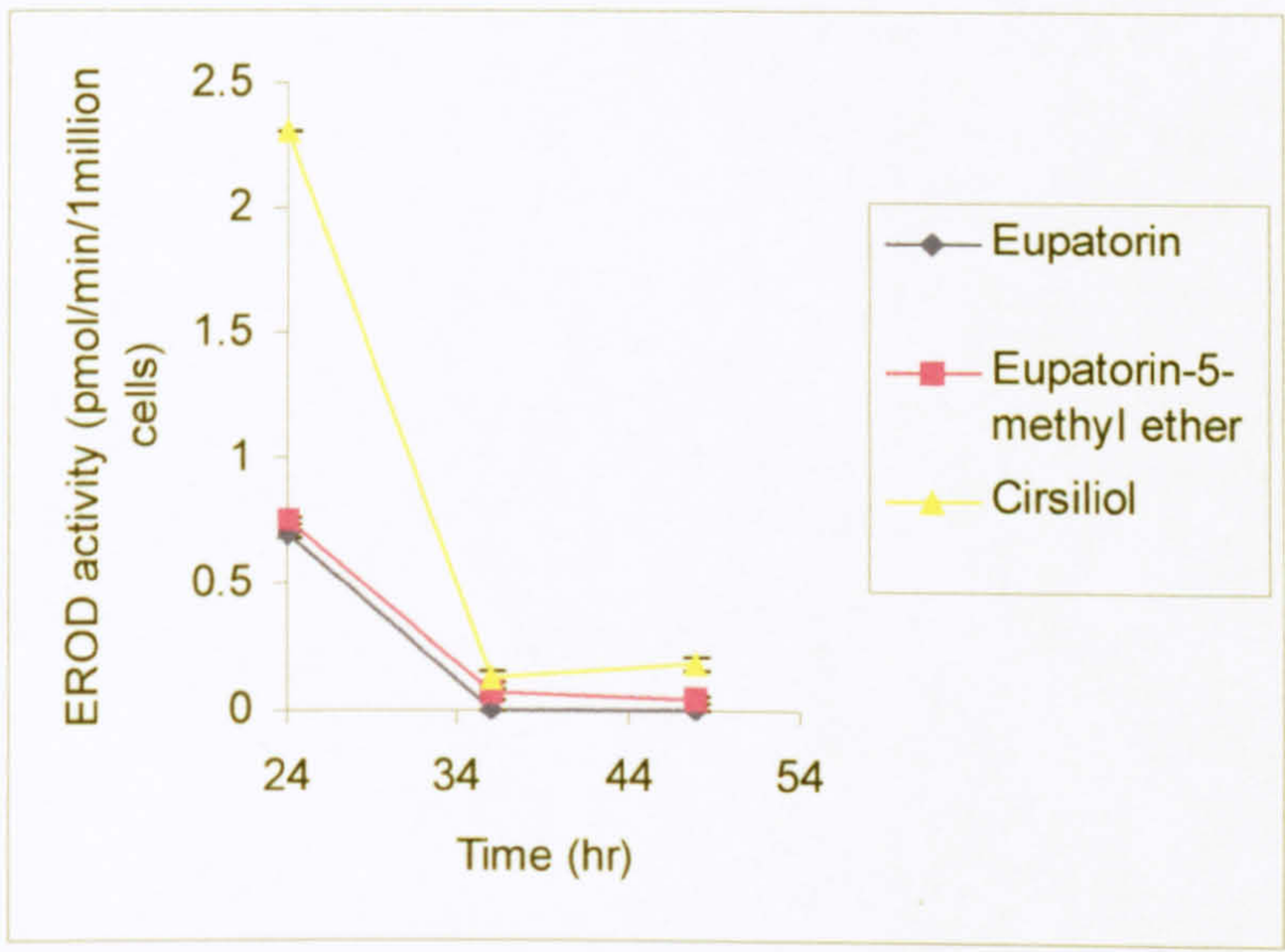
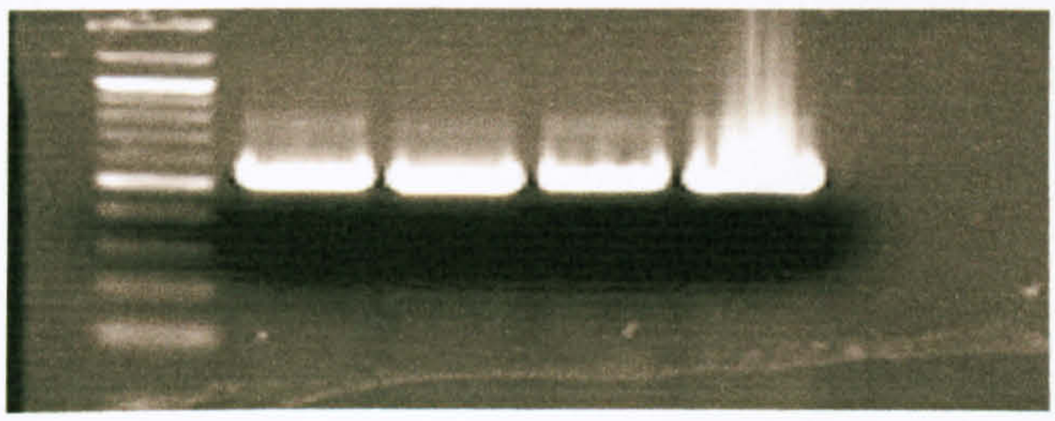


Figure 7.12 Time dependence of CYP1 enzyme induction from flavonoids (2.5 μM) in MCF7 cells. Experiments were carried out in triplicate. Error bars represent mean ± SD.

Since EROD activity is not specific for a certain CYP1 isoform, the effect of some of the above mentioned natural products on the transcription of *CYP1A1* and *CYP1B1* in MCF7 cells was investigated. The cells were treated with the compounds as before for 24 hours and the amount of mRNA was measured by semi-quantitative RTPCR. Eupatorin caused an increase in the mRNA levels of both *CYP1A1* and *CYP1B1* at the higher concentrations (5 μM) (Figure 7.13).

a)



b)

c)

Figure 7.13 The effect of eupatorin on the transcription of *CYP1B1* and *CYP1A1* in MCF7 cells. a) *β-actin*; lane 1: 100bp ladder, lane 2: 0.1% DMSO, lane 3: TCDD(10nM),lane 4 and 5: 1 and 5μM eupatorin. b) *CYP1A1*; lane 1: 100bp ladder, lane 2 : 0.1% DMSO, lane 3: TCDD(10nM),lane 4 and 5: 1 and 5μM eupatorin. c) *CYP1B1*; lane 1: 0.1% DMSO, lane 2: 1μM eupatorin, lane 3: TCDD(10nM),lane 4: 5μM eupatorin, lane 8: 1kbp ladder.

Cirsiliol caused a dose dependent increase in the mRNA levels of *CYP1B1* and at 2.5 and 1.25 μM an increase of *CYP1A1* mRNA (Figure 7.14). At 5 μM cirsiliol had no major effect on the transcription of *CYP1A1* (Figure 7.14). This compound is acting by inducing *CYP1A1* and *CYP1B1* at low and high concentrations respectively.

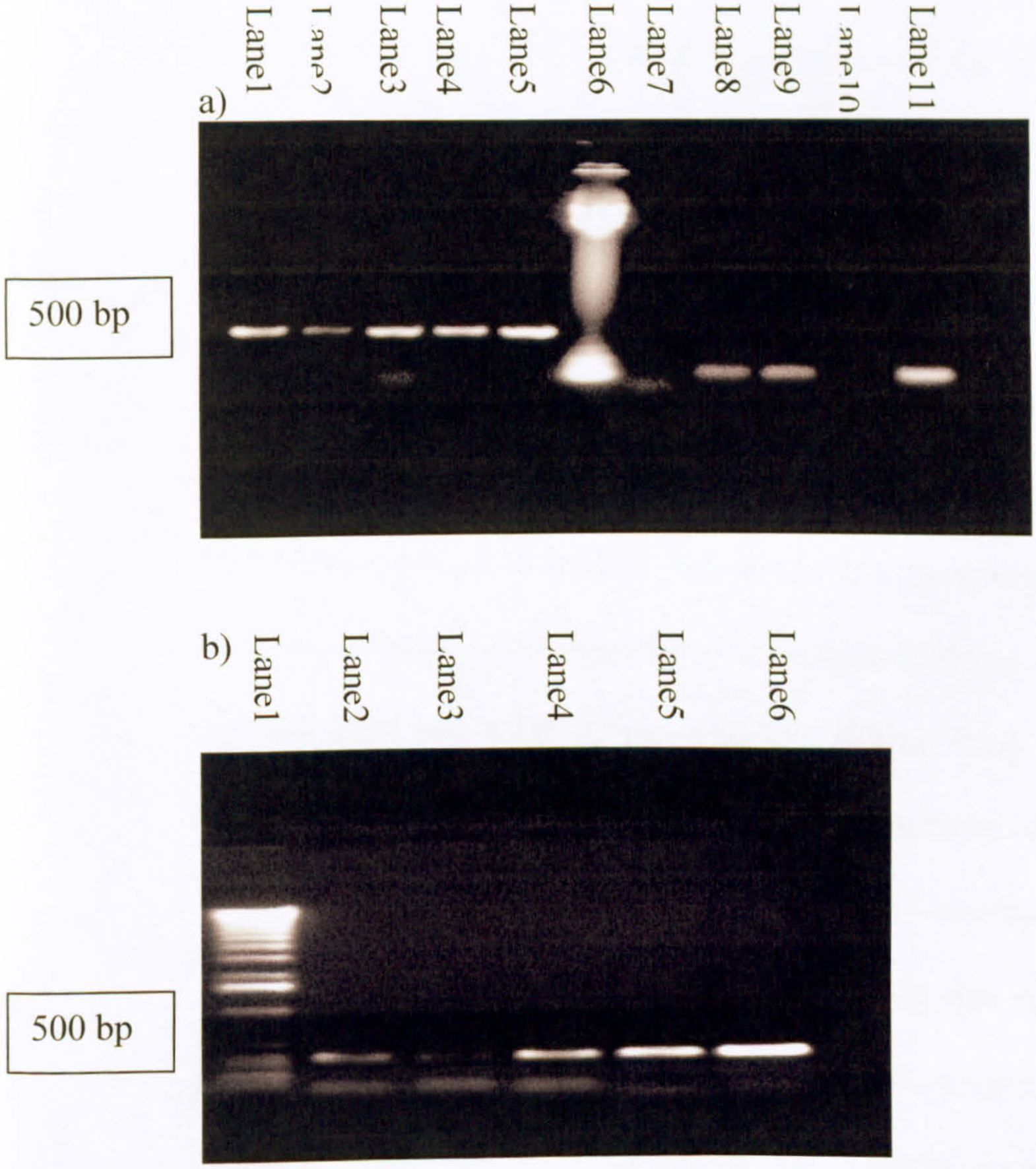


Figure 7.14 Cirsiliol induction of *CYP1A1* and *CYP1B1* mRNA in MCF7 cells. a) left to right; *β-actin* 0, 1.25, 2.5, 5 μM cirsiliol, TCDD (10 nM), 1kb ladder, *CYP1A1* 0, 1.25, 2.5, 5 μM cirsiliol, TCDD (10 nM). b) left to right; *CYP1B1* 1kb ladder, 1.25 μM cirsiliol, 0.1% DMSO, 2.5, 5 μM cirsiliol, 10 nM TCDD.

7.2.4 Natural dietary flavonoids can induce their own metabolism. Role of CYP1B1 in the prevention of cancer

As mentioned in chapter 1 several studies in the past have examined the chemopreventative properties of flavonoids through different mechanisms of action. One of the most extensively studied mechanisms is the inhibition of steps in a carcinogen activation pathway. The phorbol esters such as TPA (phorbol 12 –O-tetradecanoate 13-acetate) have been used from the early 70s to study the mechanism of carcinogenesis. Several compounds such as the flavonoids can influence the phorbol ester-induced carcinogenesis in cell lines or animal models and this mechanism of action has been used to explain their chemopreventative properties. A study (Shih et al., 2000) has shown that flavonoids such as quercetin and green tea polyphenols can interact with the AP-1 (activator protein-1) binding site of the human *CYP1A2* enhancer and thus influence TPA induced AP-1 and *CYP1A2* transcription. In this study the authors found that green tea polyphenols inhibited TPA induced luciferase activity in a cell line derived from HepG2 stably transfected with the *CYP1A2* enhancer, whereas quercetin activated luciferase activity in a dose dependent manner, suggesting that dietary chemopreventative agents may work through different pathways to modulate gene expression.

In the present study it is shown by EROD activity that dietary flavones induce the expression of CYP1 enzymes in MCF7 cells. MCF7 cells were chosen, because previous studies have examined the induction of TCDD and flavonoids in this cell line extensively (Dohr et al., 1995, Ciolino et al., 1998). MDA-MB 468 cells have a constitutive expression of CYP1 enzymes as shown by EROD activity in chapter 5, hence this would

interfere with the induction process. Furthermore the flavonoids of interest would be metabolised in that cell line and if there was any induction process occuring at all, it would be difficult to relate it to the parent compound or the active metabolites.

Flavones with no hydroxy substitution on the B ring, such as chrysin and baicalein did not have a major effect on CYP1 enzyme induction. Apigenin with a hydroxy substitution on the 3′ position also showed little effects. Further hydroxy or methoxy substitution on the B ring e.g. luteolin and diosmetin, seemed to enhance the CYP1 inducing potential of the flavones. Methoxy substitutions on the A ring of the flavones had a profound effect on induced EROD activity. The compounds eupatorin, eupatorin-5-methyl ether and cirsiliol were strong inducers of CYP1 enzyme expression. Genkwanin with a methoxy substitution at position 7 showed a small increase in EROD activity.

Furthermore the effects of eupatorin and cirsiliol on the trancription of *CYP1A1* and *CYP1B1* were examined. Eupatorin induced *CYP1A1* and *CYP1B1* mRNA levels at 5 μM. The increase in *CYP1B1* mRNA considerably higher than the one observed for *CYP1A1*. Cirsiliol caused a concentration dependent increase in *CYP1B1* mRNA levels. The greatest effect was seen at 5 μM. In contrast the increase in *CYP1A1* mRNA from cirsiliol did not seem to be concentration dependent and was completely abolished at 5 μM.

Unfortunately it cannot be concluded from these studies, whether or not the induction of CYP1 enzyme expression and the transcription of *CYP1A1* and *CYP1B1*, noticed from the above flavones are AhR mediated. However it is known that the AhR favours compounds that are hydrophobic and have Van der Waals dimensions of 14 x 12 x 5 Å (Lu et al., 1996). AhR also favours a stereocoplanar structure like the one observed for

the strongest agonist TCDD. Flavones have both these chemical characteristics. It has been shown from some studies that flavonoids can be antagonits of the AhR, by competing with the binding of TCDD (Lu et al., 1996). Some of them such as tamarixetin exhibit weak agonistic effects (Ashida et al., 2000). 3′-amino-4′-methoxy flavone induces *CYP1A1* mRNA levels and binds with high affinity to the AhR. It did not inhibit TCDD induced transformation of the AhR (Roblin et al., 2004). Tamarixetin has a 4′ methoxy group (-OCH_3). We showed that diosmetin and eupatorin, which both contain methoxy groups induce CYP1 enzyme expression. Therefore it might be assumed that the mechanism of their induction could be AhR dependent.

Diosmetin has already been reported to induce CYP1A1 expression through the AhR in MCF7 cells (Ciolino et al., 1998). Ciolino and coworkers have done extensive studies on the agonistic/antagonistic effects of some of the most commonly encountered flavonoids on the AhR. They investigated the effects of flavonoids such as galangin, quercetin, kaempferol, diosmetin, diosmin and the polyphenolic curcumin on the induction of CYP1A1 expression and activation of AhR in MCF7 cells, in a series of different studies (Ciolino and Yeh, 1999, Ciolino et al., 1999, Ciolino et al., 1998, Ciolino et al., 1998). They showed that quercetin is a strong inducer of CYP1A1 expression to the protein and mRNA level and a ligand for the AhR. Kaempferol did not induce CYP1A1 activity but was shown to be a ligand of the AhR. Kaempferol and quercetin also inhibited DMBA (dimethylbenzanthracene) induced CYP1A1 EROD activity. Galangin, which is the flavonol equivalent of chrysin, was a weak ligand of the AhR and caused a small increase in *CYP1A1* mRNA levels at the higher concentrations (5 μM). It also inhibited DMBA and TCDD induced EROD activity and cytotoxicity in MCF7 cells. Curcumin, diosmin

and diosmetin were shown to activate the AhR and induce *CYP1A1* mRNA levels and EROD activity in MCF7 cells. Diosmin, the glucoside of diosmetin, was the most potent inducer amongst all the flavonoids examined in this series of studies. The proposed hypothesis of this group, regarding the mechanism of action of these natural products, is that they can act in two different ways. Flavonoids which are agonists of the AhR induce CYP1A1 and phase II expression and prevent cancer by promoting detoxification of PAHs. In parallel flavonoids which are antagonists of the AhR compete with PAHs binding affinity and therefore reduce the metabolism of PAHs from CYP1A1 to genotoxic metabolites.

This proposed chemopreventative model does not explain why dietary flavonoids would induce CYP1 enzyme expression in tumors, if they act as inhibitors of these enzymes. Moreover, if CYP1 enzymes contribute to the progression of cancer through metabolic activation of their substrates, dietary flavonoids would be more likely to cause cancer than prevent it. It was shown in chapters 4 and 5 that the flavones examined are substrates of CYP1 enzymes and underlined that the EROD assay cannot distinguish between competitive inhibition and substrate specificity (chapter 3). Hence it is proposed that dietary flavonoids can induce the expression of CYP1 family enzymes in tumors and they can be selectively metabolised to a cytotoxic molecule by the tumor cell. These results demonstrate that this mechanism of action is possible at concentrations (2.5 μM) close to the physiological levels of circulating flavones in plasma (see chapter 1 section 1.).

Even though the AhR was identified initially as a major component of the carcinogen activation pathway, such as the one observed for TCDD, induction of CYP1A1 and

CYP1B1 expression accompanied by AhR translocation to the nucleus has been shown to be the mechanism of action of the antitumor agent DF203 (2-(4-aminophenyl)benzothiazoles with 3′-methyl substitution) (Loaiza-Perez et al., 2002). DF203 aquires its cytotoxicity by activating its own metabolism from CYP1A1, which eventually leads to induction of DNA damage and cell growth inhibition. In that regard DF203 may be regarded as a prodrug whose basis for efficacy is selective induction of CYP1A1 to produce an active species, which has antitumour effects.

Induction of CYP1A1 and CYP1B1 in tumor cells does not necessarily aid the formation of mutagenic metabolites from PAHs and thereby promote carcinogenesis as was commonly believed. In fact this induction process could be the mechanism of action of a chemotherapeutic agent (e.g. DF203). Natural products can be classified as dietary chemopreventative prodrugs, inducing their own metabolism through cytochrome P450 enzymes, such as CYP1B1 and CYP1A1. The present results also show that eupatorin and cirsiliol induced *CYP1B1* more selectively than *CYP1A1*, compared to the PAH TCDD, in the MCF7 cell line. This reinforces the possible role of CYP1B1 acting as a tumour suppresor enzyme; through metabolic activation of dietary prodrugs.

8. CONCLUSION/FUTURE WORK

Role of CYP1 enzymes in carcinogenesis

The cytochrome P450 enzymes CYP1A1 and CYP1B1 have been linked by several authors with the promotion of cancer, due to their ability to activate PAHs and related compounds. CYP1B1 is only expressed in cancer cells as reported by various studies and this was initially seen as further indication that this enzyme causes cancer.

The results presented in this thesis are contradictory to the above hypothesis. Based on these data, it is proposed that CYP1A1 and CYP1B1 should be termed "cancer preventative" enzymes, because of their selective expression in tumor cells, which makes them a target for a molecular chemotherapeutic approach. It was demonstrated in *in vitro* enzyme and cell based assays(chapters 4,5 and 6) that this type of approach is possible and can yield molecules, which are causing selective inhibition of cancer cell growth. The prodrugs, which are not converted in normal cells, have very little or no effects on normal cell growth and development.

The role of flavonoids in the prevention of cancer

Flavonoids have been termed "cancer preventative" , because of their various types of bioactivity towards tumor cells and the different mechanisms of their action have been explained in more detail in section 1.2.3. The hypothesis, which is based on the results presented in this thesis, provides a more conclusive concept about the relationship between diet and chemoprevention, as opposed to the previously developed theories (antioxidants, antimutagenic etc.). This is based on a very simple observation: The chemical structures of several of the flavonoid subclasses can be overlayed on the structure of oestradiol. This means that in theory most of the flavonoids encountered in

the diet are substrates for CYP1B1 and CYP1A1. CYP1A1 and CYP1B1 catalyse aromatic hydroxylation reactions on the A or B ring of the flavonoids. To date most of the studies, which have examined this type of bioconversion on flavonoids or related compounds from cytochrome P450s have concluded that it results in increased activity of the metabolites compared to the parent compound. The studies presented here confirm these findings. Therefore dietary flavonoids can stop the development of tumors after bioactivation by CYP1A1 or CYP1B1.

The compounds investigated in this thesis are all natural products, and present a vast variety of structurally related analogues. Such molecules, could be lead compounds for the development of synthetic prodrugs with similar or even greater activity than the natural products examined.

Further evidence about a possible link between diet and chemoprevention is provided (Chapter 4,5 and 6). Flavonoids present in the diet, are likely to be metabolised from tumor cells and their metabolic products cause selective cell death (Chapter 5). In addition the dietary flavonoids can induce their own metabolism in tumors where CYP1B1 and CYP1A1 active protein is not expressed (chapter 7).

The drawbacks and limitations of the experiments presented in this thesis are listed below:

1) The 24 hr time point has been used in the past from Ciolino (1998, 1999) to investigate the induction process in MCF7 cells. This time point seems appropriate for protein expression of CYP1 enzymes, as evident by EROD activity results, however as far as CYP1 enzyme mRNA levels are concerned, a shorter time window is required, since

mRNA induction precedes protein induction. This was demonstrated by Dohr et al., 1995 using TCDD as an inducer. The maximum time point where mRNA levels of *CYP1A1* and *CYP1B1* were examined was 10 hrs, whereas EROD assay was conducted at the 24 hr time period.

Real time PCR can also be performed to produce a more accurate measurement of the amplification reaction and instead of providing an endpoint result, it would ensure continuos monitoring of the reaction. Real time PCR has been used to investigate induction of CYP1A1 and CYP1B1 in MCF7 cells by indirubin (Spink et al., 2003) as well as in HepG2 and MOG-G-CCM by 3-methylcholanthrene (McFadyen et al., 2003).

3) The induction of CYP1 enzyme expression from natural products can be investigated by HPLC, which would be the most sensitive assay, since it would directly relate to functional activity and neither to protein nor to mRNA levels. However the limitation of this technique in the present thesis, which was also seen in the metabolism studies in cells, is that no compound examined was selectively metabolized by a certain CYP1 enzyme. All compounds investigated were substrates to a greater or smaller extent by all three CYP1 enzymes. The oestradiol hydroxylation assay has been employed in the past by some studies (Hayes et al., 1996,Spink et al., 1998, Spink et al., 2003) to investigate expression of CYP1A1 or CYP1B1 in cells, since CYP1B1 will hydroxylate in the 4 position and CYP1A1 in the 2. However even this assay is not entirely selective and CYP1A2 has been shown to also hydroxylate to a smaller extent at the 2,4 and 16 position (Badawi et al., 2001).

4) A CYP1B1 selective substrate would also overcome the lack of specificity seen in the case of the Gentest antibody. Alternatively monoclonal or polyclonal antibodies can be

raised and this has been done by Murray et al., 1997,1999, but this would be a difficult and time consuming challenge. A different protocol with Gentest CYP1B1 antibody cannot be ruled out though, which would include overnight blocking and 3 hr exposure to the primary antibody as it has been shown by Chua et al., 2000. This would in theory reduce background reactivity. Moreover microsomes from MDA-MB 468 cells could be prepared to provide a relatively purer sample, which would contain less non specific proteins.

5) Other techniques could be employed to study CYP1 protein expression, such as immunofluoresence or flow cytometry using a secondary FITC labeled antibody, but the limitations are again that they rely on the specificity of the primary antibody. For example flow cytometry has been employed to study the acetylation levels of histone H3 or H4 and the results were more sensitive than those seen in western blotting techniques (Ronzoni et al., 2005).

6) Finally the DNA analysis used in this thesis has to be combined with another technique to provide more insight on the mechanism of action of each compound. CDK assays can be performed to investigate if a compound is directly inhibiting CDKs. Moreover upregulation of cell cycle inhibitors such as p21 or p53 can be investigated with western blotting, as it has been shown by Pan et al., 2002. G2/M arrest could be further investigated by using the Comet assay, which will show whether a compound is binding on DNA and causing single or double strand breaks. However given the structures of the flavones investigated in this thesis, the most likely explanation for eupatorin and cirsiliol G2/M arrests could probably be that they or their metabolites are antimitotic agents. This can be investigated by using confoqal microscopy to examine

tubulin damage by using a α-tubulin primary antibody, as it has been shown by Kobayakawa et al., 2004.

Possible ideas for improvement and extension of these studies are listed below:

Flavones as substrates for CYP1 enzymes

1. The cell line used as the main tool of CYP1 mediated metabolism is MDA-MB 468. This cell line expresses both CYP1A1 and CYP1B1 as shown by the literature and the present studies. It is important to select a cell line, devoid of any CYP isoforms. Then this cell line can be transfected to express constituvely either CYP1A1 or CYP1B1 or other CYPs to further investigate metabolism in a cell-based system.

2. The flavonoids examined in the enzyme and cell based assays, should be screened against a human P450 panel, with the exception of diosmetin. It is necessary to ensure, at least for the compounds which show promising activity, that they are not metabolised or activated in the liver, before reaching the tumour site. Flavonoids can be screened in enzyme assays against various liver CYPs or alternatively incubated with a liver homogenate and analysed by HPLC.

3. Metabolism of flavonoids is not limited to the liver. As mentioned briefly in the introduction, flavonoids can be directly absorbed from the small intestine. The flavonoid glycosides can be hydrolysed in the intestine by the enzyme lactase phlorizin hydrolase (LPH), which is present on the brush border surface. The resulting aglycones can be further conjugated with sulfate and glucuronide groups and either excreted into the intestinal lumen or carried back to the liver via enterohepatic circulation. It would be of interest to examine the metabolism of the flavonoid glycosides by enzyme assays or

CaCo-2 cell culture model. Briefly CaCo-2 cells can be used as a model to predict intestinal absorbtion of a drug, by measuring initial concentration and the concentration of the drug after it has penetrated through the monolayer.

4. A further characterisation of the products of CYP1 enzyme conversion is necessary. For example we could combine HPLC with mass spectrometry. Mass spec data will give more conclusive information on the identification of the metabolites, resulting from P450 metabolism.

5. Finally other flavonoids such as the catechins and the anthocyanidins can be investigated as possible CYP1 family enzyme substrates.

Biological activity of natural flavones and their CYP1 conversion products

6. The biological activity of the metabolites has to be examined more thoroughly to provide supportive evidence on the CYP1A1/CYP1B1 bioactivation process. Tyrosine kinase assays can be performed, to compare the ability of the metabolites to inhibit tyrosine kinase enzyme activity with the parent compound. This can be assayed on a total tyrosine kinase assay kit or specific tyrosine kinases can be extracted and used in such assays, e.g. luteolin is an inhibitor of EGFR.

7. Some of the flavonoids examined, such as eupatorin, showed promising activity in MDA-MB 468 cells, in various different assays. It is necessary to ensure whether this effect is due to CYP1A1 or CYP1B1. Acacetin or α-napthoflavone are not selective and will inhibit both CYP1A1 and CYP1B1. SiRNA can be used to knock down either CYP1A1 or CYP1B1. MTT assays can then be performed on the knock down cell lines.

Cell viability can be related to activity and hence we can speculate whether CYP1B1 will produce more cytotoxic metabolites of a compound than CYP1A1. Once the siRNA experiment has been completed animal studies can also be performed to show an *in vivo* representation of this type of molecular cancer therapeutic approach (P450 activated prodrugs).

Regulation of CYP1 activity by dietary flavones

8. The induction of eupatorin, eupatorin-5-methyl ether and cirsiliol was not fully investigated. Whether these compounds induce their own metabolism via the AhR still remains unknown. RTPCR studies were not carried out for eupatorin-5-methyl ether. Moreover a time profile of *CYP1B1* and *CYP1A1* mRNA levels after treatment with 2.5 or 5 μM of these compounds will be of particular interest.

9. Why would CYP1B1 and CYP1A1 mRNA be present when the protein is not there? Both of these enzymes were expressed to the mRNA level (chapter 7) in MCF7 cells, however they were completely inactive to the protein level, unless induced with TCDD or a natural inducer, as shown by EROD and HPLC (chapters 5 and 7). By using a potent natural or chemical inducer of CYP1A1 and CYP1B1 the transcriptional and translational regulation of these two enzymes could be investigated in MCF7 cells. Or is it that the protein is present and subject to postranslational modifications, which activate it to the form that we detect by EROD or HPLC?

The role of CYP1 enzymes in cancer prevention

In this thesis a variety of data is presented that, combined, make a strong case for a role of the CYP1 enzymes in the prevention of cancer, rather than in the development of cancer. It is a well established fact, that a diet rich in fruit and vegetables, can play an important role in the prevention of cancer. In this thesis it is shown that several flavones, that commonly occur in a diet that is rich in fruits and vegetables, are substrates for CYP1 enzymes and preliminary data suggest strongly that the bioconversion products, or the activated products, selectively inhibit growth of CYP1 expressing cells.

Much work still needs to be done in order to get a full understanding of the mechanisms that underly the CYP1-activated prodrug model, but the data presented so far justify that this model is considered as a serious addition to the existing models that have tried to explain the widely accepted role of flavones in cancer prevention.

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